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ALTERED GLUCOSE TOLERANCE WITH HISTOLOGICALLY NORMAL ISLETS FOLLOWING REPEATED SMALL DOSES OF ALLOXAN[1]

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DUNN AND COWORKERS (1943) observed beta cell necrosis in the pancreatic islets of rats following the administration of alloxan subcutaneously (200–400 mg./kg.) in a single injection. Such animals became permanently diabetic if they survived the initial toxic effects of the chemical. Shipley and Rannefeld (1945) noted the decreased glucose tolerance of rats receiving 25 mg./kg. doses intravenously. Hyperglycemia and glucosuria were not, however, exhibited by these animals.[3] To correlate this altered glucose tolerance with some histological evidence, if present, of beta cell impairment was the purpose of the experiment being reported.

EXPERIMENTAL

Five groups of albino rats of mixed sex were used. Animals weighed about 100 grams at the start of the experiment, and were weighed every 10 days while under observation. They were fed Purina Fox Chow ad libitum. The stock of rats of the Department of Anatomy colony, although not inbred by brother-sister matings, is quite uniform genetically.

Alloxan[4] in 4 per cent aqueous solution was injected intravenously every other day in doses of 20 mg./kg. of body weight. One group of rats received 2, one 4, and a third group 10 doses. A fourth group received a single dose of 30 mg./kg., and a fifth group received no al-

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[2] Senior Research Fellow, USPHS.

[3] Single dose of 30 mg./kg. given intravenously to rats in this laboratory induced beta cell necrosis with persistent hyperglycemia and glucosuria.

[4] Alloxan monohydrate—Eastman Kodak Co.

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loxan. Glucose tolerance tests were conducted 10 days after the last injection of alloxan by withdrawing a 16 hour fasting blood sample, injecting 0.5 g./kg. glucose intravenously in 20 per cent solution, and drawing blood samples at one-half, one, and 2 hour intervals. By the

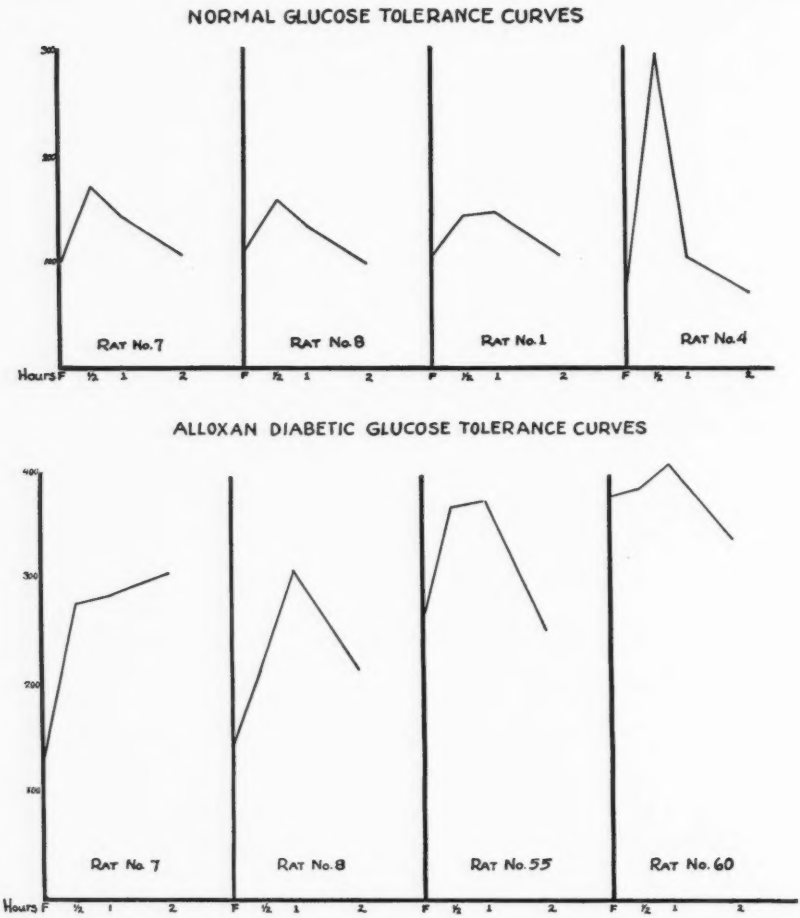


FIG. 1. Comparison of glucose tolerance curves in untreated rats and rats made diabetic by a single intravenous injection of alloxan (30 mg./kg.).

end of 2 hours the blood sugar should return to the fasting blood sugar value, or below, if the rat is normal (Fig. 1). Blood sugars were determined using a modified Folin-Wu procedure (Folin, 1929).

All groups of animals were carried on the experiment until 18 days after the last injection of the 10 dose group. All animals were sacri-

TABLE 1. DATA PRESENTING CORRELATION BETWEEN HISTOLOGY OF THE PANCREATIC ISLETS AND GLUCOSE TOLERANCE IN ALLOXAN-INJECTED AND UNTREATED RATS.

Group	Number of Animals	Alloxan	Glucose Tolerance	Islets of Langerhan
One	4	2 injections 20 mg./kg.	Normal	Normal
Two	10	4 injections 20 mg./kg.	Altered	Normal
Three	7	10 injections 20 mg./kg.	Altered	Reduction of B* cell number
Four	4	1 injection 30 mg./kg.	Altered	Few B* cells present
Five	4	None	Normal	Normal

* Beta

TABLE 2. AVERAGES AND RANGES OF BLOOD GLUCOSE LEVELS DURING GLUCOSE TOLERANCE TESTS OF UNTREATED RATS AND RATS RECEIVING MULTIPLE INTRAVENOUS INJECTIONS OF 20 MG./KG. OF ALLOXAN

No. of Rats	FBS*	½ hour	1 hour	2 hours
		Untreated Rats		
6	103 (96–110)	179 (126–294)	130 (104–148)	100 (70–116)
		2 Injections		
4	84 (70–104)	162 (124–190)	92 (78–108)	86 (76–94)
		4 Injections		
10	98 (80–110)	195 (156–230)	167 (140–188)	145 (132–160)
		10 Injections		
7	90 (66–118)	212 (156–342)	165 (124–206)	142 (126–174)

* Fasting blood sugar.

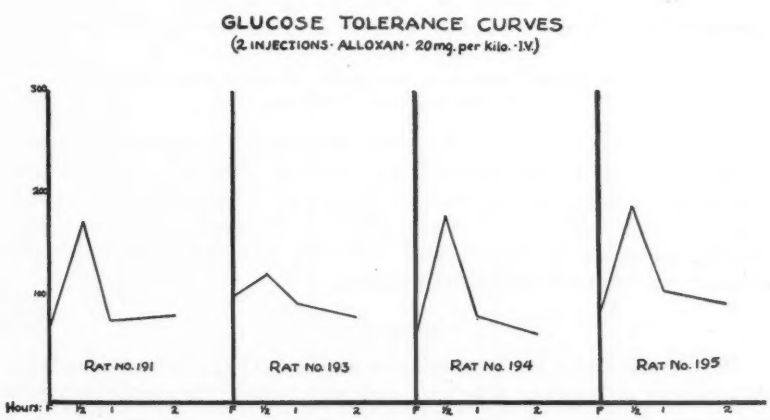

GLUCOSE TOLERANCE CURVES
(2 INJECTIONS · ALLOXAN · 20 mg. per kilo. · I.V.)
300
200
100
RAT NO. 191
RAT NO. 193
RAT NO. 194
RAT NO. 195
Hours: F ½ 1 2 F ½ 1 2 F ½ 1 2 F ½ 1 2

FIG. 2. Glucose tolerance curves of rats receiving two intravenous 20 mg./kg. injections of alloxan. These curves are normal.

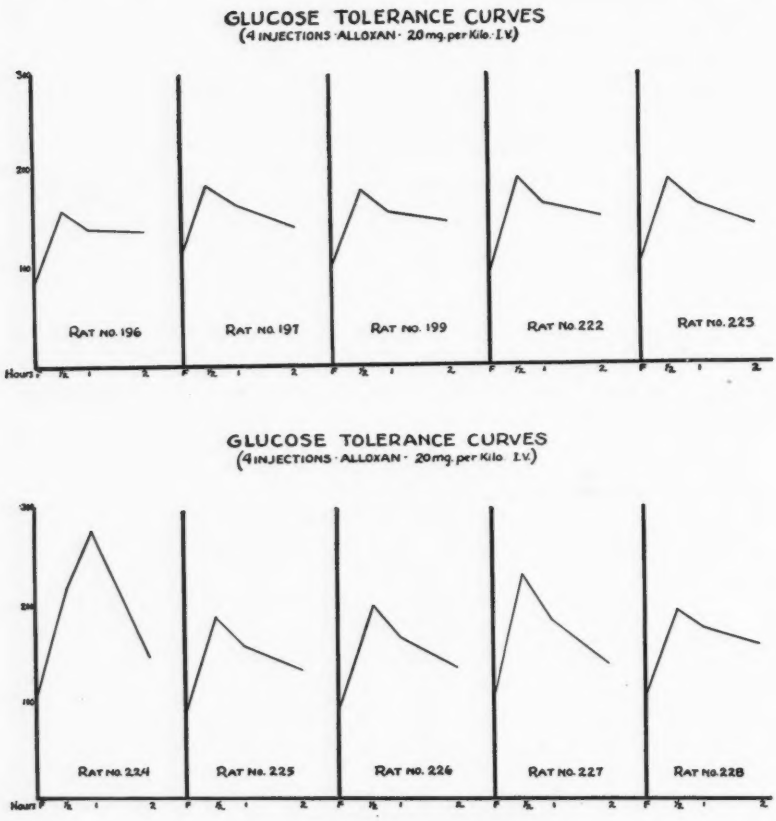


FIG. 3. Altered glucose tolerance curves of rats which received 4 intravenous 20 mg./kg. doses of alloxan.

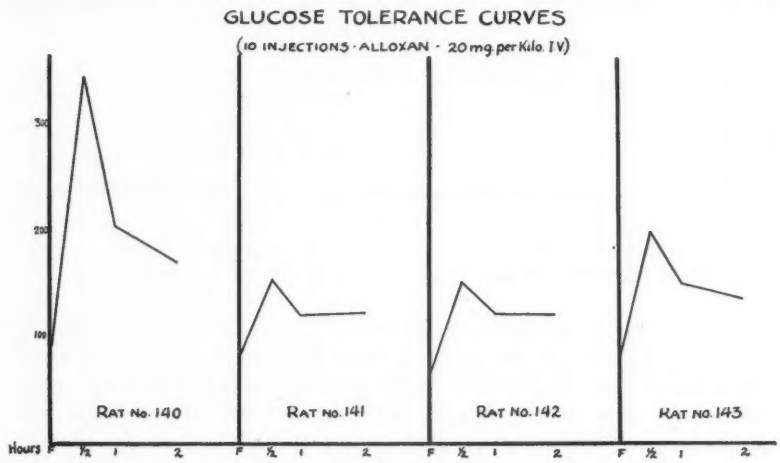
ficed without starvation at the same time under uniform conditions. Samples of pancreatic tissue were fixed in Zenker's, Bouin's and Lavdovsky's fluid. Tissue was sectioned at 4 microns and stained with Gomori's method (Gomori, 1943) or Heidenhain's iron hematoxylin. Every tenth section was mounted, the alpha and beta cells counted, and the size of the islet noted.

RESULTS

In animals receiving 2 injections of 20 mg./kg. of alloxan, neither abnormality of glucose tolerance nor what could be considered any significant alteration in the islets of Langerhans was noted (Tables 1 and 2, Figs. 1 and 2). In the group of rats that received 4 injections of 20 mg./kg. of alloxan impairment in glucose tolerance was found

(Fig. 3), the blood level of sugar being higher than normal at the end of 2 hours following the injection of 20 per cent glucose solution. Fasting blood sugar levels were normal in these rats. Microscopic examination of the islets (Figs. 6 and 7) revealed no apparent changes in the beta cells, and no decrease in their number (Table 3) as compared to normal islet tissue. The beta cells appeared to contain their full complement of granules (Fig. 8).

Rats that received 10 injections of 20 mg./kg. of alloxan showed



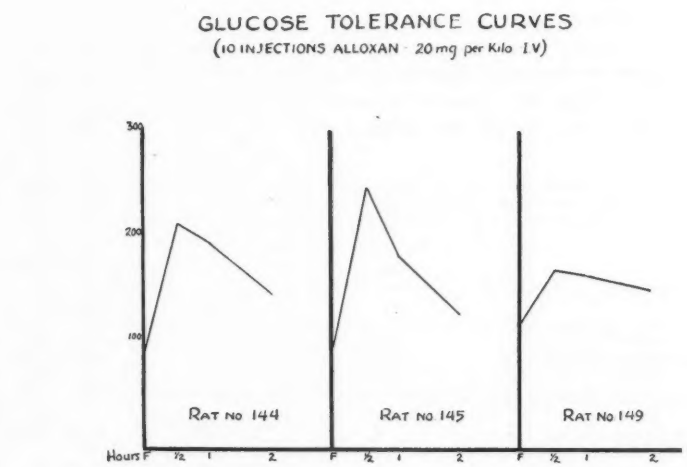


FIG. 4. Altered glucose tolerance curves of rats which received 10 intravenous 20 mg./kg. doses of alloxan.

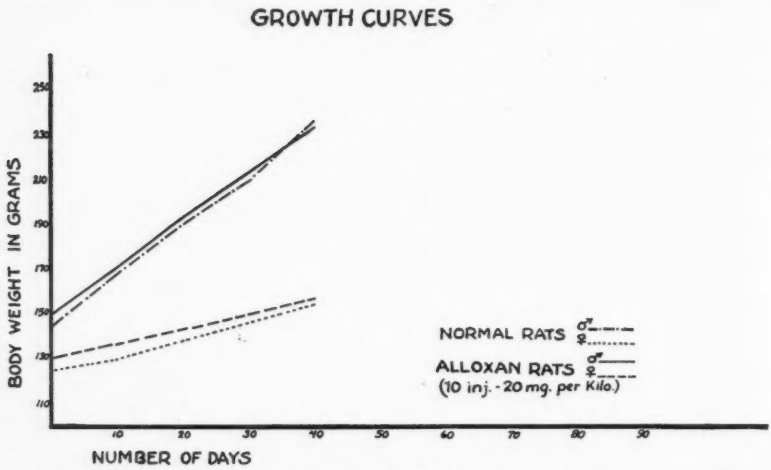


FIG. 5. Comparative growth curves of untreated male and female rats and rats of both sexes which received 10 intravenous 20 mg/kg. doses of alloxan.

glucose tolerances similar to those found in animals receiving four 20 mg. doses (Fig. 4). In the islets of these animals there was beta cell degeneration (Fig. 9), however, with a reduction in the average number of beta cells for each islet (Table 3). The per cent of beta cells ranged from 0 to 30, as compared to normal islets where there were equal numbers of alpha and beta cells. Hyperglycemia and glucosuria did not occur in the 10 dose animals. Control rats showed normal glucose tolerances (Fig. 1).

The islets of rats receiving a single dose of 30 mg./kg. of alloxan exhibited extensive beta cell degeneration. Some of the islets contained only alpha cells (Fig. 10). These rats showed persistent hyperglycemia and glucosuria, and decidedly altered glucose tolerances (Fig. 1).

TABLE 3. PER CENT DISTRIBUTION OF ALPHA AND BETA CELLS IN THE PANCREATIC ISLET TISSUE OF RATS RECEIVING MULTIPLE INTRAVENOUS INJECTIONS OF 20 MG./KG. DOSES OF ALLOXAN, OR ONE 30 MG./KG. DOSE, OR NO ALLOXAN

Group	Number of islets	Average per cent distribution of islet cells	
		Alpha	Beta
One 2 injections alloxan 20 mg./kg.	4	50	50
Two 4 injections alloxan 20 mg./kg.	8	50	50
Three 10 injections alloxan 20 mg./kg.	8	70–100	0–30
Four 1 injection alloxan 30 mg./kg.	4	70–100	0–30
Five Controls	4	50	50

Diabetic animals (30 mg./kg.) had a stunted growth and a body weight below that of normal rats of the same age (Fig. 5). In the groups receiving 2, 4, or 10 injections of alloxan normal growth was maintained (Fig. 6).

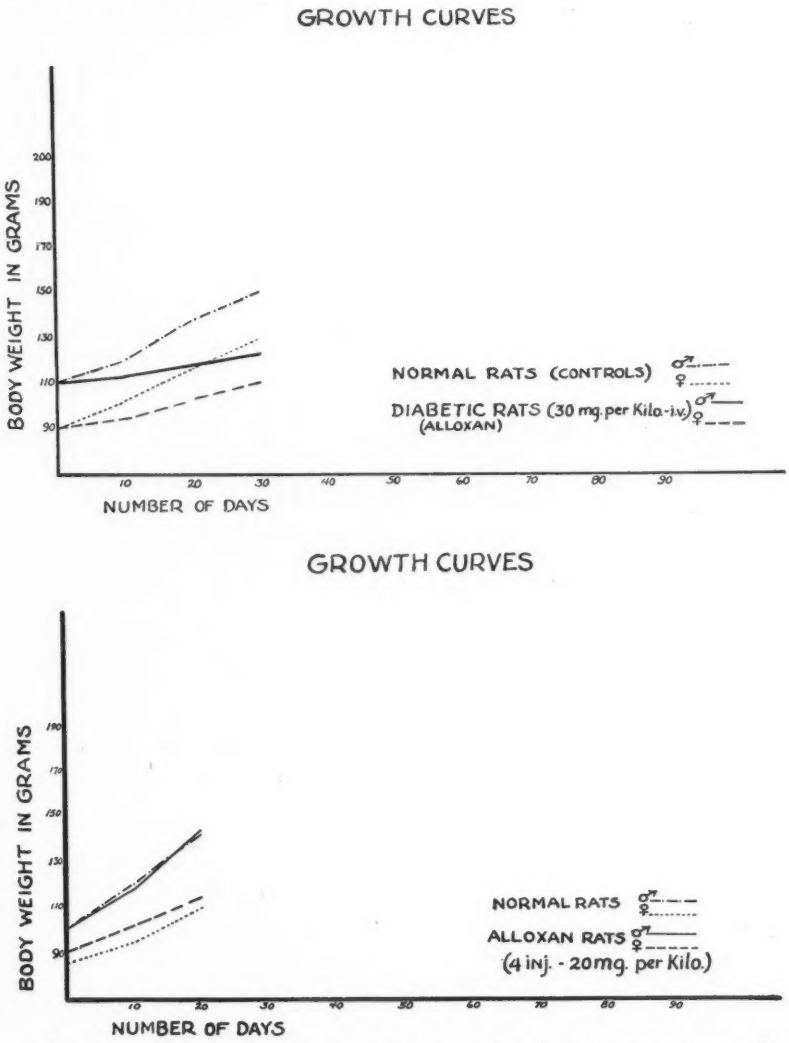


FIG. 6. Comparative growth curves of untreated and alloxan-treated rats. The upper graph compares the growth of normal rats and rats made diabetic by a single intravenous injection of 30 mg./kg. of alloxan. The lower graph compares the growth of normal rats and rats which received 4 intravenous injections of 20 mg./kg. of alloxan.

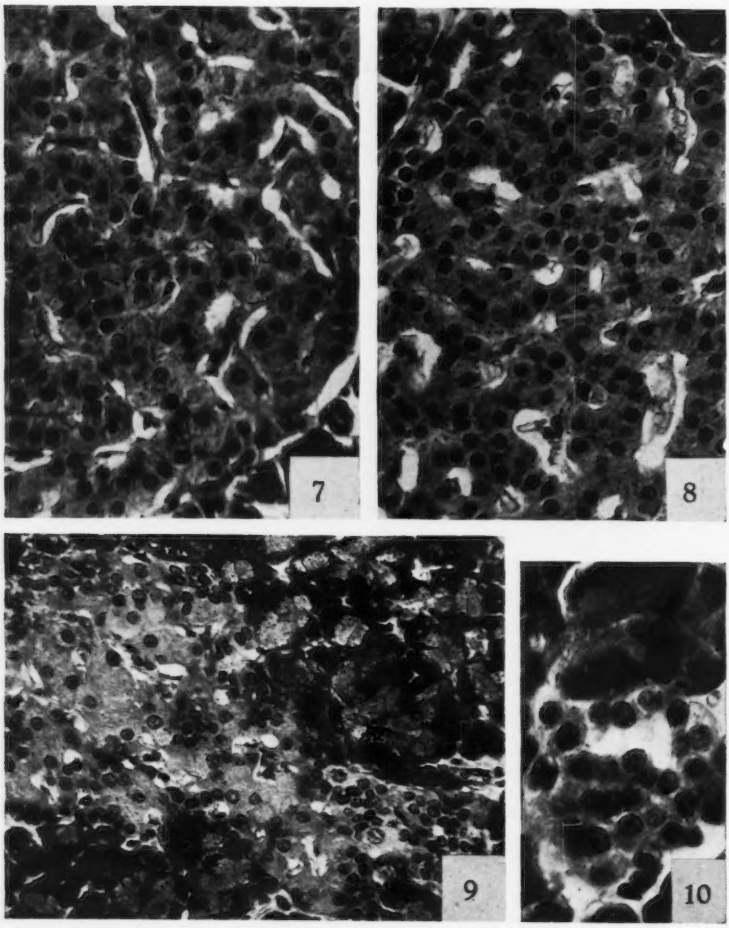


PLATE I

FIG. 7. Islet of Langerhans from an untreated rat. Beta cells compose most of this area, alpha cells being present in only the periphery of the islet in the lower right portion. Lavdovsky-Iron Hematoxylin. Photomicrograph ×300.

FIG. 8. Islet of Langerhans from a rat which received 4 intravenous doses of 20 mg./kg. of alloxan. This animal had an altered glucose tolerance curve. Islet tissue normal. Lavdovsky-Iron Hematoxylin. Photomicrograph ×300.

FIG. 9. Islet of Langerhans from a rat which received 10 intravenous doses of 20 mg./kg. of alloxan. This animal had an altered glucose tolerance curve, but showed neither hyperglycemia nor glycosuria. Growth was normal. Disorganization of islet structure with reduced number of beta cells. Lavdovsky-Delafield's Hematoxylin. Photomicrograph ×150.

FIG. 10. Islet of Langerhans from a rat which received a single intravenous 30 mg./kg. dose of alloxan. This animal was clinically diabetic. Islet small consisting of only alpha cells. Lavdovsky-Iron Hematoxylin. Photomicrograph ×600.

DISCUSSION

In rats receiving four 20 mg./kg. injections of alloxan, alteration in glucose tolerance without significant histological evidence of beta cell damage was found. Functional alteration of beta cells, that appear intact microscopically, would seem to result from small, repeated doses of alloxan. Animals which exhibit neither hyperglycemia nor glucosuria, but whose beta cells are functionally altered, represent excellent test objects for the influence of other hormones, such as thyroid and adrenal cortical, on the islets. With ten 20 mg./kg. injections of alloxan, although the beta cells were actually reduced in number and the structural alteration was extreme (Fig. 9), glucose tolerance was essentially the same as in the 4 dose animals (Figs. 3 and 4). It is interesting that the rats receiving ten 20 mg./kg. doses were not clinically diabetic, although injection of a single 30 mg./kg. dose resulted in permanent clinical diabetes with degeneration of most of the beta cells (Fig. 10).

The functional disturbance of beta cells in the absence of any decided cytological alteration (with the methods used) that might be considered significant represents a situation which is perhaps analogous to that observed in at least one-fourth of the pancreases of human diabetics (Bell, 1947).

SUMMARY

Functional alteration of the beta cells of the islets of Langerhans, in the absence of obvious histological damage, resulted from four 20 mg./kg. doses of alloxan given intravenously. The degree of functional inpairment was no greater when, following ten 20 mg./kg. doses, beta cell necrosis was present. Neither treatment induced clinical diabetes, although permanent diabetes ensued following a single intravenous dose of only 30 mg./kg.

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INSULIN HYPERSENSITIVITY FOLLOWING THE ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE[1]

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THE ADMINISTRATION of adrenal cortical hormone inhibits or prevents the release of pituitary adrenocorticotrophic hormone (ACTH) which normally follows subjection of an animal to noxious agents or stressful conditions (Ingle, 1938; Sayers and Sayers, 1947). It has been demonstrated that cortical steroids with an oxygen on C_{11} as well as those of the desoxy type have an inhibitory action upon pituitary adrenocorticotrophic activity. Since these two major types of cortical steroids appear to be qualitatively distinct in their metabolic actions, one group influencing protein and carbohydrate metabolism and the other group electrolyte metabolism, it has been suggested that the cortical steroids act directly on the adenohypophysis rather than indirectly through some product of their metabolic action or through their deficiency (Sayers and Sayers, 1947). A necessary corollary of this "direct action" hypothesis is the following: Administration of desoxycorticosterone acetate (DCA), by inhibiting the adrenocorticotrophic activity of the adenohypophysis and in turn the secretory activity of the adrenal cortex, should lead to a disturbance in carbohydrate metabolism characteristic of a deficiency of those steroids having O on C-11. The hypothesis is supported by the experiments presented in this paper which demonstrate that DCA-treated rats are more sensitive than untreated animals to the hypoglycemic action of insulin.

MATERIALS AND METHODS

Male rats from the Sprague-Dawley farm, weighing 220 to 450 grams, were employed. In order to demedullate the rats, the adrenals were enucleated through paravertebral incisions. Before DCA was implanted, a period of two weeks was allowed for regeneration of the cortex from the capsule in series I and II, and a period of one week in series III. Each series was divided equally into two groups; one group served as a control and into each member of the other group six 15-mg. pellets of DCA were implanted subcutaneously. The pellets were distributed over the region of the flanks. The amount of DCA

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[2] Fellow of the American Bureau for Medical Aid to China.

absorbed was calculated from the difference between the original weight of the pellets and their weight at the time of autopsy. The average amount absorbed per rat per day was 1.7 mg.

The insulin sensitivity tests were conducted from the second to fourth weeks after the DCA implantation. The animals were fasted 12 hours and then regular insulin was administered intraperitoneally in a dose of 0.5 unit per Kg. Blood samples for sugar analysis were taken from the tail vein immediately preceding the administration of insulin and at hourly intervals thereafter for four hours. Blood sugars were analyzed by Nelson's (1944) modification of the Somogyi method.

The aqueous[3] and lipid[4] extracts of adrenal cortex were administered intramuscularly. The ethanol preservative was removed from the aqueous extract just preceding its administration. At the time of the insulin test each rat was given cortical extract in two equal doses; the first injection was made one-half to one hour before and the second injection immediately following the administration of insulin. Each single dose of the lipid extract was one to 1.25 ml. and each single dose of aqueous extract was the equivalent of 2.5 ml. of the original aqueous-alcohol solution.

The experiments were planned so that an equal number of controls and treated animals were tested for insulin sensitivity on any one particular day. Usually three controls and three DCA-treated rats were tested on the same day. Each experiment designed to test the therapeutic effectiveness of cortical extract in DCA-treated animals included two controls, two DCA-treated rats and two DCA cortical extract-treated rats.

Additional experiments were performed on completely adrenalectomized rats in order to obtain an estimate of the relative sensitivity of the DCA-treated demedullated rats to insulin.

RESULTS

Insulin sensitivity of DCA-treated demedullated rats. The results of series I, a total of 19 rats, are presented in Figure 1. The data for series II and III, a total of 22 rats, have been combined in Figure 2. The dose of insulin used, 0.5 unit per Kg., is smaller than that required to produce the same reduction in blood glucose in rats with intact adrenal medullas. Demedullated rats were employed in order to avoid the complication of epinephrine discharge which occurs after insulin administration.

There was no significant difference between the fasting blood glucose levels of the untreated demedullated rats and the DCA-treated demedullated rats. This was true of all three series of animals.

It is apparent from Figure 1 (series I) and Figure 2 (series II and III) that administration of DCA to demedullated rats markedly increases their sensitivity to insulin. In series I, five of the 10 DCA-treated animals died in hypoglycemic coma; the remaining five had insulin tolerance curves which clearly indicated increased sensitivity to insulin. None of the control animals died. The tolerance curve of

[3] Adrenal Cortex Extract (Upjohn).
[4] Lipo-Adrenal Cortex (Upjohn).

only one of the nine control animals was in the zone of the curves of the DCA-treated animals. The incidence and severity of coma and convulsions in the surviving DCA-treated animals were greater than in the untreated controls. One of the rats of the DCA group died at a time when the blood sugar level was above what might be considered critical; the explanation of this is not apparent.

In series II and III (data combined in Figure 2), the results confirm those obtained in series I. Three of the 8 DCA-treated rats died

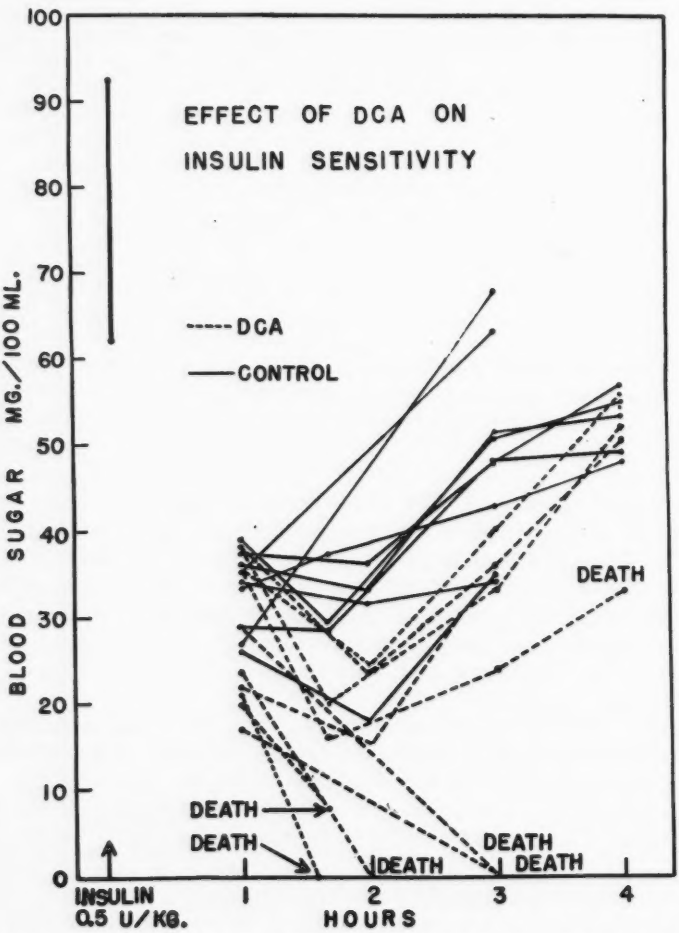

FIG. 1. Series I. The effect of DCA administration in demedullated rats upon insulin sensitivity. Regular insulin was injected intraperitoneally in a dose of 0.5 unit per Kg. at zero time. The vertical line at zero time represents the range in fasting blood sugar of all rats in series I.

in hypoglycemic coma following insulin administration whereas no deaths occurred in the control group. The tolerance curves of the two groups overlapped but slightly. Again the incidence and severity of convulsions and coma were greater in the surviving DCA-treated animals than in the controls. From Figure 2 it can be seen that cortical extract increased the resistance of the DCA group to insulin to

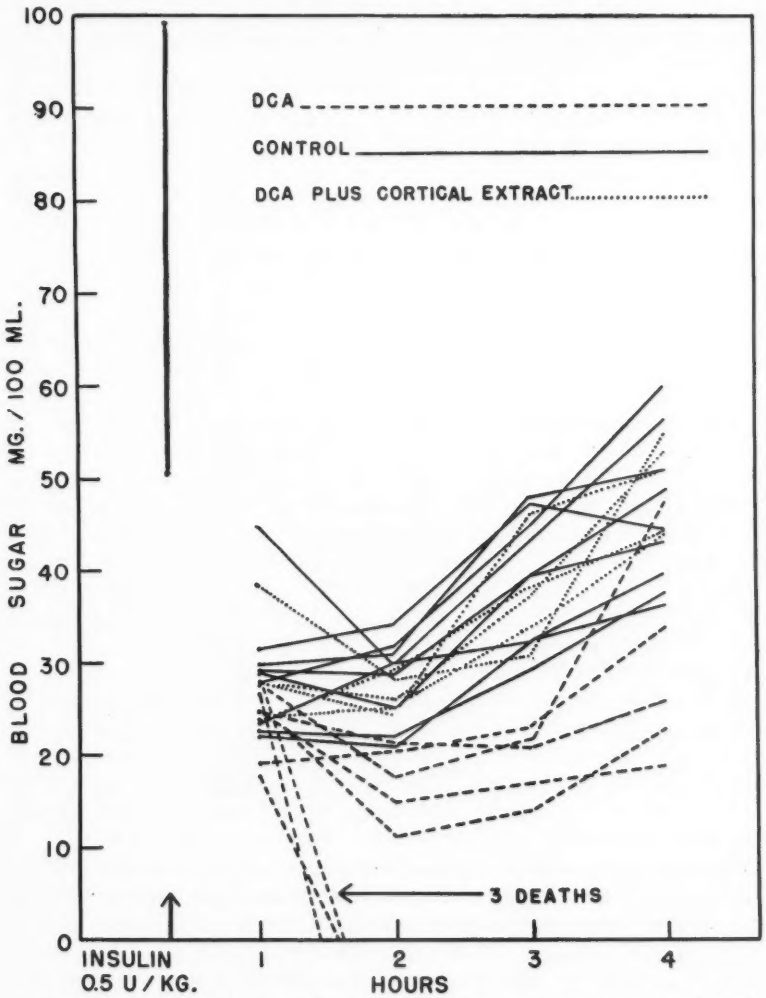

FIG. 2. Series II and III. The effect of DCA administration in adrenal demedullated rats upon insulin sensitivity; the antagonistic action of cortical extract. Regular insulin was injected intraperitoneally in a dose of 0.5 unit per Kg. at zero time. The vertical line at zero time represents the range in fasting blood sugar of all rats in series II and III.

such a degree that their tolerance curves fell into the normal zone. Furthermore, the clinical symptoms of the animals given DCA and cortical extract were indistinguishable from those of the demedullated controls.

Effect of adrenalectomy on insulin sensitivity. An estimate was made of the relative sensitivity of the DCA group to insulin in comparison

TABLE 1. RELATIVE INSULIN SENSITIVITY OF ADRENALECTOMIZED, DEMEDULLATED AND DCA TREATED-DEMEDULLATED RATS

Dose of insulin units per Kg.	Group	Blood sugar mg. per 100 ml.				
		Fasting level	Hours after insulin administration			
			1	2	3	4
0.25	demedullated	67	31	35	44	63
		59	32	32	40	51
	demedullated plus DCA	54	38	42	43	50
		55	23	20	30	42
	adrenex	48	24	18	16	22
		62	24	18	25	34
0.50	demedullated	83	30	31	48	51
		75	31	34	47	45
	demedullated plus DCA	70	28	Died		
		65	28	Died		
	adrenex	64	9.5	Died		
		66	22	Died		
1.00	demedullated	68	11	5	6	Died
		70	19	19	50	51
		74	24	19	21	34
		60	23	21	26	34
		62	15	25	18	20
	demedullated plus DCA	71	15	Died		
		66	12	Died		
		62	15	Died		
		62	19	Died		
		55	16	—	17	21
	adrenex	71	4	Died		
		69	2	Died		
		65	8	Died		
		63	17	Died		

with that of completely adrenalectomized animals. Table 1 presents the results of this study. All members of the three groups,—the demedullated control, the demedullated DCA-treated, and the adrenalectomized animals,—survived following the administration of 0.25 unit of insulin per Kg. However, the tolerance curves suggest that the adrenalectomized rats were slightly more sensitive than the DCA-treated animals. Only the controls survived the administration of 0.5 unit of insulin per Kg. Following the administration of 1.0 unit of insulin per Kg. only one member of the control group died, whereas

all the DCA-treated animals succumbed except one; all four adrenalectomized rats died following such treatment. Again the tolerance curves indicate that the adrenalectomized animals are more sensitive to insulin than are the DCA-treated rats.

DISCUSSION

Demedullated rats were employed in these studies in order to eliminate the possible masking of insulin sensitivity by discharge of epinephrine from the adrenal medulla. The cortex rapidly regenerates from the capsule following enucleation of the adrenal and when regeneration is complete the animal appears to be normal except for its response to those stressful situations in which the elaboration of epinephrine represents an essential phase of the attempt to maintain homeostasis (see Ingle [1944] for a discussion of the problem of the resistance of the adrenal demedullated animal).

The results of this study clearly indicate that the administration of DCA produces an increase in the sensitivity of rats to insulin. The degree of sensitivity approaches but is not quite as severe as that of the adrenalectomized animal. It is reasonable to suppose that the increased sensitivity to insulin which follows administration of DCA is a result of a relative deficiency of cortical steroids having an O on C-11. The evidence is admittedly indirect but the explanation is in accord with our present knowledge of the influence of hormones upon carbohydrate metabolism. A number of published reports have appeared which may also be interpreted to indicate that DCA produces an impairment of carbohydrate metabolism characteristic of a deficiency of those steroids with an oxygen on C-11. For example, administration of DCA is said to increase the tolerance of diabetic patients to glucose (Köhler and Fleckenstein, 1941, 1942; Winnett *et al.*, 1940). McGavack *et al.* (1941) have demonstrated that DCA influences the response of normal human subjects to oral glucose; the administration of DCA caused "an early higher rise and a marked depression approaching hypoglycemic levels in the fourth and fifth hour." Furthermore, chronic treatment with DCA impairs the ability of the rat to mobilize glucose when subjected to a variety of nonspecific types of stress (Selye and Dosne, 1942).

The effect of DCA upon electrolyte metabolism has been definitely established. Unfortunately, it is not possible at the present time to reach a final conclusion regarding the influence of this steroid on carbohydrate metabolism. Does its action differ in degree only from those steroids with an O on C-11, or is DCA qualitatively distinct in its metabolic activity? The answer to this question has an important bearing on the interpretation of the results which have been presented in this paper. Under the conditions of these experiments DCA acts as an "antidiabetogenic" substance. Furthermore, even in large doses DCA has no activity as measured by the glycogen deposition test

(Olson *et al.*, 1944; Pabst *et al.*, 1947). However, two groups of investigators have reported that DCA has a "diabetogenic" action. Ingle and Thorn (1941) found that doses of 1, 2 or 5 mg. per day of 17-hydroxy-11-dehydrocorticosterone were followed by enhanced glycosuria in partially depancreatized rats, whereas similar doses of DCA had no effect. However, 10 mg. of DCA did produce a definite exacerbation of the glycosuria. Wells and Kendall (1940) demonstrated that DCA, although not quite as effective as Compound E, very definitely increased the glycosuria of the phlorhizinized-adrenalectomized rat. These experiments support the view that large doses of DCA have a diabetogenic effect, *i.e.*, a metabolic action similar to that of the cortical steroids with an O on C-11. However, it is important to note that this so-called "diabetogenic" action has been demonstrated using large doses of DCA in experiments employing glycosuria as an index of "diabetogenic" action. It is possible that large doses of DCA produce this "diabetogenic" effect through a renal mechanism, that is, by inhibiting the reabsorption of glucose by the renal tubule. This possibility takes on added significance in the light of the experiments of Wells and Kendall (1940) who noted that Compound E markedly increased the glycosuria of phlorhizinized-adrenalectomized rats and at the same time prevented the collapse, convulsions and death which frequently occurred in the untreated controls. On the other hand, DCA produced a definite although less marked glycosuria, but was entirely ineffective in preventing coma, convulsions and death. This may be interpreted to mean that DCA did not benefit the adrenalectomized animals by elevating the blood sugar but rather acted synergistically with phlorhizin to inhibit reabsorption of glucose at low blood sugar levels. This possibility is currently being subjected to experimental test by renal clearance studies.

It is obvious that further experimental work will be necessary in order to establish definitely whether DCA has any action at all on carbohydrate metabolism similar to that of the steroids with an O on C-11. The results of the least complicated test, namely, the glycogen deposition test, would seem to indicate that DCA is qualitatively distinct in its metabolic actions from those steroids with an O on C-11.

It has been demonstrated that the administration of cortical hormones prevents or inhibits the discharge of ACTH which otherwise follows subjection of an animal to a variety of nonspecific types of stress (Sayers and Sayers, 1947). It would appear reasonable to suppose that the great variety of non-specific stresses increases pituitary adrenocorticotrophic activity by a common mechanism, namely, by increasing the requirement of the peripheral tissue cells for cortical hormones. Since the inhibitory effect on the pituitary is exerted by both the desoxy type cortical steroids and those having an O on C-11,

steroids which differ markedly or even qualitatively in their metabolic actions, it has been suggested that the steroids act directly on the adenohypophysis, rather than indirectly through some product of their metabolic activity or through their deficiency. A corollary follows from this "direct action" hypothesis. Administration of DCA, by suppressing pituitary adrenocorticotrophic activity and in turn the secretory activity of the adrenal cortex, should result in a cortical hormone imbalance characterized by an excess of DCA and a deficiency of those normally secreted cortical steroids which influence carbohydrate metabolism. The experiments presented in this paper support such a view.

The concept of "direct action" of cortical steroids on the adenohypophysis appears to us to be the most reasonable explanation of the experimental facts at present available. However, other interpretations are possible. For example, DCA might produce the effect described by a direct inhibitory action on the secretory activity of the adrenal cortex. Although evidence in favor of such an action has been presented by Greep and Deane (1947), the experimental results of Ingle *et al.* (1938) and Sayers and Sayers (1947) are against such a possibility.

The structural similarity of desoxycorticosterone and the steroids with an oxygen on C-11 presents the possibility of competitive inhibition. It is conceivable that these two types of steroids could interfere with each other in their actions upon a specific target cell. However, the fact that the administration of DCA causes atrophy of the adrenal cortex (Villela, 1943) is strong evidence in favor of an action, direct or indirect, through the adrenal cortex itself.

The authors in collaboration with Dr. Dixon Woodbury have recently demonstrated that the elevation of both the electroshock seizure threshold and the plasma sodium of intact rats treated with DCA can be reduced to normal levels by ACTH administered concomitantly with the DCA. These results may be interpreted to mean that at least part of the action of DCA in the intact animal is a result of the suppression of pituitary adrenocorticotrophic activity with a consequent reduction in the secretory activity of the adrenal cortex. They lend support to the concept of "direct action" presented above.

Selye and Pentz (1943) have demonstrated that DCA administered in large doses to rats on a high sodium chloride intake causes hypertension, nephrosclerosis, cardiac lesions and periarteritis nodosa, and believe these experiments support their concept that certain chronic degenerative diseases in man are a result of hypercorticism. However, according to the present study, DCA treatment leads to the development of a state of steroid hormone imbalance characterized by an excess of a compound influencing electrolyte metabolism and a deficiency of steroids with an O on C-11. The role of the deficiency state as well as that of the excess DCA must be con-

sidered in arriving at conclusions regarding the etiological role of DCA in the production of pathological conditions of the cardiovascular system.

SUMMARY

The administration of DCA to adrenal demedullated rats resulted in insulin hypersensitivity. The DCA-treated animals were more sensitive than untreated controls but less sensitive than adrenalectomized rats. The results have been interpreted to mean that DCA produces a state of relative deficiency of those cortical steroids with an O on C-11. A number of possible mechanisms whereby DCA can affect insulin sensitivity has been presented. A direct inhibitory action of DCA on pituitary adrenocorticotrophic activity appears to be the most reasonable explanation of the facts at present available.

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A NEW PROCEDURE FOR THE DETERMINATION OF THE ANTIDIURETIC PRINCIPLE IN THE URINE[1]

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IT IS GENERALLY accepted that the hormone of the neurohypophysis secretes a hormone which plays an important role in the water economy of the organism. This antidiuretic hormone is presumably excreted in part in the urine, the antidiuretic activity of which has been found to parallel the need of the organism for the hormone (Gilman and Goodman, 1937). Thus during dehydration resulting from forced abstinence from water or in various clinical conditions (cirrhosis of the liver with ascites (Ralli, *et al.*, 1945), the nephrotic stage of glomerulonephritis (Robinson and Farr, 1940), eclampsia (Ham and Landis, 1942,) etc.), the antidiuretic potency of the urine, is enhanced considerably over normal. In dehydration, structural changes are also noted in the neurohypophysis, which are suggestive of increased activity of this tissue (Gersh, 1939).

The available methods for the determination of the antidiuretic principle in the urine are laborious. Although the hydrated rat is very sensitive to small doses of the hormone, losses are apt to occur in the available procedures used in concentrating the urine in the commonly used method of Burn (1931) and its modifications. These procedures consist in concentrating 24-hour collections of urine by evaporation and dialysis of the residue. However, the available evidence (Ham and Landis, 1942; Ralli, *et al.*, 1945; Donaldson, 1947) indicates that the membranes used for the latter procedures are not entirely impermeable to the hormone which may, therefore, be partially lost.

The present paper describes a procedure for concentrating the hormone in urine which is simple and does not require dialysis. The hormone derived from larger volumes of urine may be concentrated in the final extract with the exclusion of toxic urinary constituents which is not possible by previous methods. It is thus possible to detect even small amounts of the antidiuretic hormone by concentrating correspondingly larger volumes of urine.

Posterior pituitary solutions manifest a chloruretic as well as an

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antidiuretic action. The antidiuretic action of urinary concentrates, on the other hand, is not accompanied by a corresponding chloruretic effect which might be taken as evidence against the identity of the hormone with the principle occurring in the urine. The probable cause of this anomaly has also been elucidated.

METHODS

For comparative purposes, the procedures used by previous workers for extracting the hormone were utilized. Twenty-four-hour collections of urine were acidified with 3 per cent acetic acid to about pH 6, placed in Visking membranes and concentrated at room temperature by suspension in the air under an electric fan. The concentrated urine was then dialyzed for 6 hours and evaporated further to a volume of about 80 cc. This residue, after filtration, was brought to a volume of exactly 100 cc. with distilled water and injected in doses of 1 cc. per 100 gm. of body weight into the test rats. The bioassay procedure of Burn (1931) as modified by Ralli, *et al.* (1945) was used. For each determination, 6 groups of 3 rats were used, 2 groups serving as controls.

In the modified procedure which has been devised, the sample of freshly collected urine is filtered and brought to pH 4.5 to 5.0 by the addition of acetic acid. One gm. of absorbent powdered charcoal (Darco activated carbon, Grade G-60) is added for each 100 cc. of urine and the mixture agitated at intervals for several hours. It is placed in the ice-chest overnight and the supernatant urine discarded. The charcoal is collected on a Buchner funnel, washed with small amounts of distilled water to remove adherent urine, drained and transferred to a centrifuge tube. The charcoal on which the antidiuretic hormone is quantitatively adsorbed, is suspended in glacial acetic acid (5 cc. per gm. of charcoal)and agitated at intervals for several hours. The acid is separated from the charcoal by centrifugation or filtration and the hormone and other adsorbed materials precipitated by the addition of 10 volumes of a mixture of equal parts of absolute ethyl alcohol and petroleum ether. This precipitate is removed by centrifugation, the supernatant fluid being discarded. After drying in a vacuum desiccator at room temperature for at least one hour to remove the organic solvents, the precipitate is dissolved in the requisite amount of distilled water, filtered and bioassayed as indicated above.

The chloruretic action of the final extract was determined by analysis of the chloride content of the urine (50 per cent output) excreted by the rats used for assay. Chloride was determined by Stiff's (1948) modification of Sendroy's method or by the procedure of Van Slyke (1923).

RESULTS

The procedure for determining the antidiuretic activity of the urine as outlined above is based on the fact that the antidiuretic principle is adsorbed quantitatively on charcoal, eluted from the latter by glacial acetic acid and precipitated from the last-named by petroleum ether and absolute alcohol. The solubility of the antidiuretic factor in glacial acetic acid and its precipitation by petroleum ether was originally demonstrated by Kamm *et al.* (1928). By treating solu-

tions of commercial posterior pituitary extract by the above-outlined procedures, good recovery of the active principle is obtained as shown in Table 1. The data of this table were obtained by diluting commercial posterior pituitary extract with 0.9 per cent saline to give concentrations varying from 20 to 100 milliunits per cc. as shown in column 1. Aliquots of these solutions were bioassayed for their antidiuretic activity as described above in doses of 1 cc. per 100 gm. of body weight of the test rats. Similar assays were also performed on the final solutions obtained after subjecting an aliquot of the original diluted extracts to the adsorption-elution procedure outlined above. The second

TABLE 1. THE RECOVERY OF ANTIDIURETIC ACTIVITY FROM SOLUTIONS OF COMMERCIAL POSTERIOR PITUITARY LIQUID BY ADSORPTION ON CHARCOAL, ELUTION WITH GLACIAL ACETIC ACID AND PRECIPITATION WITH PETROLEUM ETHER

Concentration of posterior pituitary solution	Delay Times	
	Original extract	After adsorption, elution, etc.
milliunits per cc.	mins.	mins.
20	20	20
30	40	30
40	50	50
50	55	50
60	65	65
70	90	95
80	105	100
100	130	125

and third columns of the table give the delay times in the excretion of the water of the test rats. This delay time is the difference in time required to excrete half the water administered by the rats receiving extract as compared to the control animals receiving saline or water injections.

The method just described reduces the possibility of loss of the active principle by dialysis. Previous authors have obtained divergent results in their study of the diffusibility of the active principle (Ham and Landis, 1942; Ralli, *et al.*, 1945; Donaldson, 1947). Our own experiments using ultra-filtration and dialysis through cellophane membranes are reproduced in Table 2. The data of this table were obtained by dialyzing a solution of commercial posterior pituitary liquid containing 20 milliunits per cc. for 6 hours against running tap-water or ultrafiltering the solution under a pressure of 100 mms. of Hg through a Visking membrane. As noted in Table 2, there was only a slight loss of antidiuretic activity on prolonged dialysis. However, as seen in the results obtained on the ultrafiltrate, the membrane was definitely permeable to this principle as well as to the chloruretic factor. It would appear, therefore, that the active principle is of such a molecular size as to be on the border line as regards its capacity to pass pass through the available membranes. This would account for the divergent results of previous workers who have studied the dialyza-

bility of the active principles of posterior pituitary extracts and urine (Smith and McClosky, 1924; Gilman and Goodman, 1937; Donaldson, 1947). In any case, the avoidance of the necessity of dialysis avoids the possibility of loss of the hormone. Another advantage of the present method is that it permits one to concentrate much larger amounts of urine without undue toxicity of the final extract which is not the case when evaporation and dialysis are used, as in previous methods. As shown in the subsequent paper, the method permits the demonstration of antidiuretic activity in normal urine as well as in conditions in which its concentration is greatly increased.

TABLE 2. THE EFFECT OF DIALYSIS ON THE ANTIDIURETIC AND CHLORURETIC ACTIVITY OF AN AQUEOUS SOLUTION OF COMMERCIAL POSTERIOR PITUITARY LIQUID CONTAINING 20 MILLIUNITS PER CC.

	Chloride excretion	Time for 50 per cent urinary excretion
	m.eq. per 100 g.	mins.
Controls (saline)	1.4 1.5	90 95
Original solution of posterior pituitary liquid	5.4 5.9	165 150
Same after dialysis for 6 hours	2.5 1.5	155 120
Ultrafiltrate of same	7.3 6.3	115 130

The Chloruretic Action of Posterior Pituitary Extract

As pointed out by previous workers, the principle responsibile for the antidiuretic action of urine differs in notable respects from that of posterior pituitary solution which in turn differs in action from that of the press-juice of fresh posterior pituitary glands (Ham and Landis, 1942). The most notable difference between the extract or the press-juice and the principle in the urine is the absence in the latter of the characteristic chloruretic activity manifested by the glandular preparations. This has led many observers to question the view that the urinary principle is derived from the pituitary. It is now recognized, however, that the hormone of the posterior pituitary is a single molecule which can be readily converted to moieties of the molecule which are still capable of manifesting part of the action of the original hormone (literature reviewed by Grollman, 1947). It appeared to us likely, therefore, that one could explain the lack of chloruretic action of the urinary principle by assuming that the original hormone was partially altered in the organism, perhaps through oxidation. We, therefore, oxidized commercial posterior pituitary solution containing 10 units per cc. and saline extracts of fresh glands by adding about 1 cc. of 95 per cent hydrogen peroxide to 10 cc. of extract. After allow-

ing the mixture to stand for several hours, the solution was heated to boiling and zinc dust added to destroy the excess peroxide. The resulting solution after dilution with normal saline to give a solution containing 20 milliunits per cc. was tested for its chloruretic and antidiuretic action. As shown in Table 3, the chloruretic effect is lost following oxidation without appreciable loss of antidiuretic action. These findings support the view that a similar effect occurs in the body and that the antidiuretic principle of urine, despite its lack of chloruretic activity, may be an altered product of the posterior pituitary hormone.

TABLE 3. THE EFFECT OF OXIDATION ON THE CHLORURETIC AND ANTIDIURETIC ACTIVITIES OF POSTERIOR PITUITARY SOLUTION

	Chloride excretion	Time for 50 per cent urinary excretion
	m.eq. per 100 g.	mins.
Controls (saline)	0.6	90
	1.8	100
	1.6	80
Untreated posterior pituitary solution	10.3	160
	8.6	180
	12.0	165
Oxidized posterior pituitary solution	1.2	180
	0.9	160
	1.7	165

The results of Table 3 were obtained on commercial posterior pituitary liquid. Comparable results were obtained on saline extracts of fresh glands and on extracts of desiccated posterior pituitary powder.

DISCUSSION

The procedure outlined is not only simpler and more convenient but also affords a more accurate method for the concentration of the antidiuretic principle from urine than those previously available. It has been applied to a study of hypertension as shown in the following paper with results not obtainable by the older procedures of concentration by evaporation and dialysis.

The results of the present paper on the destruction of the chloruretic action of the hormone by oxidation removes one of the chief objections hitherto raised against the view that the antidiuretic principle in urine is derived from the posterior pituitary hormone. The ease with which the hormone as it occurs in fresh press-juice is altered in its properties by chemical manipulation (Van Dyke, *et al.*, 1942) and split into pressor and oxytocic fractions and the rapidity with which injected extracts disappear from the blood stream are evidence of the lability of the hormone. One would anticipate, therefore, that the excretory product of the hormone might appear in an altered form differing in many respects from the native hormone.

The results of the present study support the view that the chloruretic action of posterior pituitary extract is due to a specific action of the hormone (Silvette, 1940; Little, *et al.*, 1947), as opposed to the view that this action is merely incidental to its antidiuretic action.

SUMMARY

A new procedure is described for concentrating the antidiuretic principle of the urine based on adsorption on charcoal, elution by glacial acetic acid and precipitation by petroleum ether and absolute alcohol. The method offers several advantages over previously available procedures.

Oxidation of posterior pituitary extract was shown to result in a loss of its chloruretic with retention of its antidiuretic activity. It is suggested that a similar reaction occurs in the organism and that this may account for the lack of chloruretic action by the antidiuretic principle of the urine.

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THE ANTIDIURETIC HORMONE IN THE URINE IN EXPERIMENTAL AND CLINICAL HYPERTENSION[1]

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HYPERTENSION, experimentally induced in laboratory animals as well as the clinical form of this disorder, is accompanied by certain changes in salt and water metabolism (Grollman, 1947). These observations suggested the desirability of a study of the excretion of the antidiuretic principle in the urine in various forms of hypertension in order to determine to what extent the altered salt and water metabolism in hypertension is accompanied by changes in the antidiuretic activity of the urine.

METHODS

The procedures outlined in the preceding paper were utilized for the determination of the antidiuretic content of the urine. Twenty-four hour specimens of urine were collected from a group of patients suffering from hypertensive cardiovascular disease who had been followed in the hypertension clinic of the Parkland Hospital. Specimens were also collected from a series of rats and dogs in which chronic hypertension had been induced at least 6 months previously by the application of a figure-of-eight ligature to the right kidney and ablation of the left kidney (Grollman, 1944). The average resting blood pressures of the human patients are given in a subsequent table. In the case of the rats, the mean blood pressures as determined daily for periods of several weeks prior to this study ranged between 150 and 200 mms. as determined by the plethysmographic method of Williams, Harrison, and Grollman (1939), In the case of the dogs, the mean arterial blood pressure as determined by direct intra-arterial puncture with a needle connected to a mercury manometer, ranged between 150 and 180 mms.

As controls, urine collections were made in a comparable manner from normotensive men, dogs, and rats and their antidiuretic activity compared with that of the urine from the hypertensives.

In the case of the human urines (Tables 1 and 2), the 24-hour specimens or aliquots of same were finally made up to a volume equivalent to 100 cc. per 24-hours excretion. One cc. of the final extract was injected for every 100 g. of body weight of the test animals. In the case of the dogs (Table 3), the entire volume of urine was concentrated as described in the preceding paper and made up to a final solution of 10 cc. In the case of the rats (Table 4),

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TABLE 1. THE ANTIDIURETIC ACTIVITY OF THE URINE OF HYPERTENSIVE PATIENTS AS BIOASSAYED ON RATS

Patient	Age	Sex	Blood pressure	Urinary excretion in 24 hours	Chloride excretion in 50% urine	Time for 50% urinary excretion
			mms. Hg.	cc.	m.eq.	mins.
J.H.	65	F	220/130	1200	2.1	165
A.W.	45	F	150/102	1400	0.8	100
G.R.	53	M	190/110	940	1.0	180
O.E.	33	M	190/130	2320	1.1	180
E.S.	44	F	180/118	1700	1.1	180
J.B.	48	M	190/110	1100	1.2	180
D.V.	49	F	170/110	2700	1.6	180
J.V.	23	M	200/140	1330	1.7	180
E.H.	45	F	154/108	1300	0.7	155
B.H.	46	F	175/140	1675	0.3	130
M.C.	24	F	194/134	1350	0.9	90
S.D.	37	F	198/120	1350	1.3	104
P.S.	60	F	170/100	700	1.0	160
B.S.	52	F	154/104	950	0.6	150
M.E.	47	F	170/110	1000	1.1	180

TABLE 2. THE ANTIDIURETIC ACTIVITY OF THE URINE OF NORMOTENSIVE SUBJECTS AS BIOASSAYED ON RATS

Subject	Age	Sex	Blood pressure	Urinary excretion in 24 hours	Chloride excretion in 50% urine	Time for 50% urinary excretion
			mms. Hg.	cc.	m.eq.	mins.
M.E.	28	M	110/70	800	2.0	130
J.V.	26	M	112/80	1750	0.7	112
L.T.	27	M	115/80	700	0.7	115
C.B.	38	M	110/80	1550	1.1	92
C.R.	23	M	115/85	1100	0.6	100
L.L.	26	M	115/80	1050	1.0	114
A.G.	47	M	100/70	1100	1.2	116
E.G.	52	F	110/75	1210	0.8	105
A.F.	36	F	108/70	1300	1.0	122

TABLE 3. THE ANTIDIURETIC ACTIVITY OF THE URINE OF NORMAL AND HYPERTENSIVE DOGS EXPRESSED AS MILLIUNITS OF POSTERIOR PITUITARY SOLUTION

Normotensive Controls			Hypertensive Dogs		
Mean blood pressure	Antidiuretic potency of urine	Amount of antidiuretic principle excreted	Mean blood pressure	Antidiuretic potency of urine	Amount of antidiuretic principle excreted
mms. Hg.	milliunits per 100 cc.	milliunits per day	mms. Hg.	milliunits per 100 cc.	milliunits per day
100	30	60	150	55	660
110	44	68	160	225	630
115	117	214	170	296	858
95	62	76	180	309	572
100	80	84	180	270	553
105	70	182	160	118	419

the entire urinary output was also concentrated but diluted only to 2 cc. The doses administered in Tables 1 and 2, therefore, correspond to 1 per cent of the daily output per 100 g. of body weight of the test rats. In the case of Table 3, the results are given in terms of the daily excretion of the antidiuretic principle. In the case of Table 4, comparable amounts of the urine from normotensive and hypertensive animals were administered.

Controls in which 0.9 per cent saline was administered in doses of 1 cc. per 100 g. of body weight of the experimental rat were always run concurrently with the urinary extracts. The control figures after saline and after

TABLE 4. THE ANTIDIURETIC AND CHLORURETIC ACTIVITIES OF THE URINE OF NORMAL AND HYPERTENSIVE RATS

Normotensive Controls			Hypertensive Rats		
Mean blood pressure	Chloride excretion	Time for 50% urinary excretion	Mean blood pressure	Chloride excretion	Time for 50% urinary excretion
mms. Hg.	m.eq. per 100 g.	mins.	mms. Hg.	m.eq. per 100 g.	mins.
100	0.9	80	180	0.9	180
110	0.7	95	170	2.8	180
90	1.6	75	200	1.4	180
105	0.6	90	160	1.6	170
100	2.1	80	150	1.5	170
95	1.8	80	210	0.6	180

known doses of posterior pituitary extract were within the range of the values reported in the preceding paper (Grollman and Woods, 1949).

RESULTS

The chloruretic and antidiuretic activities obtained from the urine of hypertensive and normotensive humans are given in Tables 1 and 2, respectively. The tables show the absence of chloruretic activity in urine and the presence of an increased antidiuretic activity of the urine of hypertensive patients as compared to normotensive individuals. Of the 15 patients cited in Table 1, the antidiuretic activity is increased in 11 cases and is in the normal range in only 4. No difference was noted in the antidiuretic action of the first 6 subjects cited in Table 2 who were non-smokers as compared to the last 3 subjects who were heavy smokers. Any stimulating effect of nicotine or of smoking on the posterior pituitary gland (Burn, Truelove, and Burn, 1945) is thus not reflected in an increased excretion of the antidiuretic principle of the urine.

In Table 3 are given the results obtained on 5 hypertensive dogs as compared to a similar number of normotensive animals. Although the concentration of antidiuretic potency of the urine from the hypertensive dogs was within the range of normal in 2 cases, the total amount of the antidiuretic principle excreted in 24 hours was increased in every case, ranging from 419 to 858 milliunits per day in the hypertensives as compared to only 60 to 214 in the normotensive

dogs. There is thus a definite increase in the amount of antidiuretic principle excreted in the urine daily in the hypertensive dog. A similar state of affairs is also observed in the case of chronic hypertension in the rat. As shown in Table 4, the urine of the hypertensive animals exerts in every case a marked antidiuretic activity while urine from normotensive animals is devoid of such action.

DISCUSSION

The present results demonstrate an increased excretion of antidiuretic principle in the urine of hypertensive patients and of dogs and rats with induced experimental renal hypertension. Hypertensive cardiovascular disease must thus be added to cirrhosis of the liver with ascites (Ralli, *et al.*, 1943), eclampsia (Teel and Reid, 1939), and acute glomerular nephritis (Robinson and Farr, 1940) in which an increased rate of excretion of this principle also has been observed. The significance of the presence of an increased amount of the antidiuretic principle in the urine in these conditions is still a matter for speculation. Griffith and his coworkers (Griffith, *et al.*, 1941; Pendergrass, *et al.*, 1947) have suggested that the posterior pituitary gland is hyperactive in some cases of hypertension and have recommended measures designed to reduce its activity. However, there is little to support the contention of a posterior-pituitary origin of hypertension (Grollman, 1947).

All of the clinical conditions cited above in which an increased excretion of antidiuretic principle is observed have in common the tendency for the retention of salt and water. It is questionable, however, if the increased antidiuretic action of the urine represents an increased rate of secretion of the antidiuretic hormone which is responsible for the altered salt and water metabolism, or merely a secondary response to the primary disturbance in kidney or liver which is responsible for the altered salt and water metabolism. The possibility also remains that the appearance of an increased amount of antidiuretic principle in the urine does not reflect any increased activity of the posterior lobe of the pituitary but rather a decreased rate of destruction of the hormone. In fact, the generally assumed role of the posterior pituitary in regulating water metabolism has been questioned (Newton and Smirk, 1934). Other evidence indicates a possible extra-pituitary origin of the antidiuretic principle found in urine (Walker, 1939).

The fact that the antidiuretic activity of the urine is increased in both clinical hypertension of man as well as in experimental renal hypertension in animals is further evidence for the probable identity of these disorders (Grollman, 1947). Frankel and Wakerlin (1943) were unable to detect an increased excretion of the antidiuretic principle in hypertensive dogs. Their failure to do so may be attributed to the methods used by these workers. The use of the procedure outlined

in the preceding paper, however, as shown in Table 3, gives ample evidence that the dog is not unique but resembles man and the rat in the excretion of an increased amount of the antidiuretic principle in the urine when rendered hypertensive.

SUMMARY

The antidiuretic activity of the urine of normotensive men, dogs, and rats has been compared with that observed in patients with hypertension and dogs and rats with experimental renal hypertension. In 15 human hypertensives, the excretion of the antidiuretic principle was increased in 11 and within the normal range in the remaining 4. In the case of 6 hypertensive dogs, the daily excretion of the antidiuretic principle varied from 419 to 858 milliunits as compared to 60 to 214 milliunits in an equal group of normotensive animals. The urine of hypertensive rats is also strongly antidiuretic while that of normotensives exerts no such action when concentrated to the same degree. The possible significance of these observations is discussed.

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THE INFLUENCE OF ASCORBIC ACID ON THE ADRENAL WEIGHT DURING EXPOSURE TO COLD

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QUEBEC CANADA

IT IS WELL KNOWN that any type of stress produces an increase in weight of the adrenals, and it is believed that the weight response in such conditions is an aspect of the functional adaptation of the adrenals to the increased requirement for cortical hormones by the organism (Selye, 1937) (Ingle, 1938, 1939); in other words, that the gain in adrenal weight, under conditions of stress, is due to an hypertrophy of the cortex with a corresponding increase in adrenal cortical hormones production, and consequently an increased resistance towards the damaging agent. It has also been shown by many authors that the enlargement of the adrenal cortex can be prevented (Ingle, 1938) (Selye, 1940) or even more that the gland may become atrophied (Selye and Dosne, 1942) when the animals under stress are treated with sufficiently high doses of adrenal cortical hormones; moreover, such a simple chemical substance as ammonium chloride has been reported to inhibit slightly the adrenal enlargement normally induced by anterior pituitary extract (Hall and Selye, 1945).

On the other hand, it has been reported quite often in recent years that a relation of some sort—still obscure—seems to exist between the ascorbic acid level of the adrenal cortex and the hormonal activity of the latter: Giroud, Santa and Martinet (1940), Giroud, Santa, Martinet and Bellon (1940), Giroud, Martinet and Bellon (1941), Giroud and Martinet (1941) have observed that not only does ascorbic acid favour the production of adrenal cortical hormones but also improves its utilization; another aspect of the same problem has been worked out by Long and his collaborators (Sayers, Sayers, Fry, White and Long, 1944), (Harkins and Long, 1945), (Long, 1947) who have found a decrease in ascorbic acid and cholesterol content of the adrenals after injection of adrenotrophic hormone or exposure to all sorts of stress including cold: those changes are seen to occur a few hours after injury; Dugal and Thérien (1947) have reported an increase in adrenal ascorbic acid for guinea-pigs and rabbits, and in other tissues for rats and guinea-pigs, during long exposures to a cold environment, when those animals get adapted to low temperatures;

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if not, the contrary occurs, namely a decrease in adrenal ascorbic acid before the animals die; the same authors also found a direct relation between ascorbic acid content of the adrenals and adaptability to cold; subsequently, Ludewig and Chanutin (1947) have also found an increase in adrenal ascorbic acid (following the initial decrease) after injury (not including exposure to cold), and Thérien and Dugal (1949) following the urinary excretion of ascorbic acid during exposure to cold were able to confirm indirectly the above findings of Long (1947), and their own (1947).

In view of all those relations between stress, on the one hand, and adrenal enlargement, adrenal ascorbic acid changes, adrenal ascorbic acid level and acclimatization, on the other, and also in view of the fact that large intake of ascorbic acid increases resistance to haemorrhagic shock (Stewart, Learmonth and Pollock, 1941) (Dosne de Pasqualini, 1946) and to cold (Dugal and Thérien, 1947), it was only natural to wonder what would be the effect of relatively large doses of ascorbic acid on the adrenal hypertrophy normally observed during short or long (Emery, Emery and Schwabe, 1940) exposure to cold.

EXPERIMENTAL

Three main experiments were conducted using white rats in the first ones and guinea-pigs in the last one.

1) Short exposure of white rats to cold.

A—*Technique.* In the first experiment, in which we wanted to study the effect of ascorbic acid on adrenal weight during a short and acute exposure to cold, 140 male white rats weighing around 200 grams were divided into four groups: two of them were exposed to a temperature of −1°C for 72 hours, the experimental group receiving daily, by intraperitoneal injections, 150 mgm. of ascorbic acid in the form of sodium ascorbate,* at the rate of three injections daily of 0.5 cc. each time, each injection being equivalent to 50 mgm. of ascorbic acid; the control group received daily an equivalent volume of a sodium chloride solution (0.9%) equally divided into 3 intraperitoneal injections. The animals were kept in individual cages and fed "Purina Fox Chow" ad libitum; the food consumption was measured and found to be the same for both groups.

Drinking water was also given ad libitum and replaced as often as necessary to avoid freezing. Two corresponding groups were kept at room temperature. All animals were sacrificed at the end of the three day period.

B—*Results.* a) A most striking and unexpected result was found: *the increase in adrenal weight,* normally seen in rats submitted to such a cold temperature as we have used and for such a lapse of time, *has been prevented by ascorbic acid:* this is clearly shown in Table 1, where

* "Redoxon forte" from Hoffman-Laroche, Montreal.

TABLE 1. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. SHORT EXPOSURE (72 HOURS) TO A TEMPERATURE OF −1°C

	Room Temperature				Cold Room				% Increase	t
	Number of animals	Weight of animals		Adrenal weight mgm.	Number of animals	Weight of animals		Adrenal weight mgm.		
		Initial	Final			Initial	Final			
Group I*	24	200.4	204.6	33.45 ±1.36‡	33	201.3	190.6	33.70 ±1.12	0.74	0.14
Group II†	25	199.9	204.2	32.47 ±0.97	33	202.1	192.6	36.09 ±0.99	*11.10*	*2.64*

* Received 150 mgm./day of ascorbic acid.
† Received 0.9% NaCl.
‡ Standard error.

the adrenals of the group receiving ascorbic acid have an average weight of 33.45 mgm. at room temperature and 33.70 mgm. at −1°C; the increase is less than 1% and is not significant (t = 0.14). On the other hand, the untreated group shows the typical increase in adrenal weight: the average values for room temperature and −1°C are respectively 32.47 mgm. and 36.09 mgm., representing an increase of 11.1% in the adrenal weight of the animals submitted to cold. This last result is statistically significant (t = 2.64).

b) Controls of each group (receiving ascorbic acid or sodium chloride) at room temperature show no significant difference in adrenal weight.

c) Survival of the ascorbic acid group in the cold room is definitely better than that of the controls submitted to the same temperature, if the experiment lasts at least a week, in the same conditions of temperature and with animals of the same weight as above (Table 2).

TABLE 2. INFLUENCE OF ASCORBIC ACID ON SURVIVAL OF RATS SUBMITTED DURING 7 DAYS TO A TEMPERATURE OF −1°C

	Number of animals	Number of dead	Survival
Group receiving 150 mgm./day of ascorbic acid	12	3	75%
Controls receiving sodium chloride	12	12	0%

It must be emphasized that after 3 days of exposure to cold in the conditions described, the difference in resistance between the two groups *cannot be appreciated* from the difference in survival, both groups *seeming* at that time equally resistant. Incidentally, it may be said here that doubtful results about the beneficial effect of ascorbic acid for resistance to cold, like the ones of Grab and Lang (1946) may be due to the use of only one criterion, the survival, during a too short period of time.

d) It is clear, from Table 3, that the control group in the cold room shows an increase in kidney weight (as compared to room temperature) but not the group receiving ascorbic acid.

2) Long exposure of white rats to cold.

A—*Technique.* In the second experiment which was performed on

TABLE 3. INFLUENCE OF ASCORBIC ACID ON KIDNEY WEIGHT. SHORT EXPOSURE (72 HOURS) TO A TEMPERATURE OF −1°C

	Room Temperature				Cold Room				% Increase	t
	Number of animals	Weight of animals		Kidney weight gm.	Number of animals	Weight of animals		Kidney weight gm.		
		Initial	Final			Initial	Final			
Group receiving ascorbic acid	25	200.4	204.6	2.141 ±0.05	32	201.3	190.6	2.057 ±0.05	−3.92	1.1
Control group receiving NaCl	25	199.9	204.2	2.076 *±0.05*	32	202.1	192.6	2.225 *±0.05*	7.17	*2.13*
Difference				3.13% (*t* =0.95)				8.1% (*t* =*2.37*)		

130 male white rats, weighing around 250 grams, two groups were exposed to a relatively mild cold of 4°C for a period of 244 days. One of the two groups received daily, by mouth, 25 mgm. of ascorbic acid, and the control group, tap water. Corresponding groups were also kept at room temperature. All other conditions were similar to those described for the first experiment.

B—*Results.* The results are the same as in the first experiment, but still more obvious.

a) The same phenomenon, that *ascorbic acid prevents the hypertrophy of the adrenals*, during exposure to cold, is still more visible here than it was for a short exposure to cold (Table 4). The average

TABLE 4. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. LONG EXPOSURE (244 DAYS) TO A TEMPERATURE OF 4°C

	Room Temperature				Cold Room				% Increase	t
	Number of animals	Weight of animals		Adrenal weight mgm.	Number of animals	Weight of animals		Adrenal weight mgm.		
		Initial	Final			Initial	Final			
Group receiving ascorbic acid	24	269	364	36.90 ±1.75	28	256.4	305.5	37.90 ±1.15	2.7	0.48
Control group (tap water)	22	270	360	36.65 *±1.89*	18	270.6	301.3	44.88 *±2.98*	22.4	*2.35*
Difference				nil				18.4% (*t* =*2.18*)		

adrenal weight for the group receiving ascorbic acid is about the same at room temperature or in the cold room. The control group, in the cold room, shows an increase in adrenal weight of over 20% (as compared with animals of the same age kept at room temperature).

b) Survival in the cold room is clearly favoured by ascorbic acid (see Table 5).

TABLE 5. INFLUENCE OF ASCORBIC ACID ON SURVIVAL OF RATS SUBMITTED 244 DAYS TO A TEMPERATURE OF 4°C.

	Number of animals	Number of dead	Survival
Group receiving ascorbic acid	35	7	80.0%
Group receiving tap water	35	17	51.4%

As a matter of fact, the ascorbic acid group submitted to cold did just as well as the controls of each group kept at room temperature.

c) Table 4 also shows that the gain in weight in the cold room is greater for the ascorbic acid group than for the control group, the per cent increases in average weight being respectively 19.1 and 11.2.

3) Long exposure of guinea-pigs to cold.

The previous results obtained on rats raised the question as to what effects ascorbic acid would have on adrenal weight of guinea-pigs, unable to synthesize that vitamin, during relatively long exposure to cold.

A—*Technique.* Two experiments were performed differing by the quantity of ascorbic acid administered daily, the initial weights of the animals (male guinea-pigs) when first exposed to cold, the temperature of the cold room and the duration of exposure. All groups received their ascorbic acid by mouth, Group II, 25 mgm. daily, Group VI, 10 mgm., and Group IX, 2 mgm. The animals were placed

TABLE 6. INFLUENCE OF ASCORBIC ACID ON ADRENAL WEIGHT. GUINEA-PIGS EXPOSED TO COLD FOR 90 DAYS*

	Room Temperature					Cold Room					% Increase	t
	Number of animals	Body weight		Average ascorbic acid mgm./gm. adrenals	Adrenal weight mgm.	Number of animals	Body weight		Average ascorbic acid mgm./gm. adrenals	Adrenal weight mgm.		
		Initial	Final				Initial	Final				
Group II	25	375.0	626.4	0.742	375.0 ±24.86	26	449.4	486.8	0.857	409.2 ±16.97	9.1	1.12
Group IX	25	391.8	654	0.182	369.7 ±20.32	23	447.7	472.3	0.167	475.5 ±18.92	28.6	3.81
Difference					1.4% (t=0.16)					16.2% (t=2.62)		

* (−8°C for 12 hours and +8°C for 12 hours every day.)

in individual cages. The food ("Purina Rabbit-Chow" was given ad libitum, measured and found to be consumed in equal quantities by the different groups exposed to the same temperature, but the consumption was greater in the cold room than at normal temperature. Drinking water was also given ad libitum.

B—*Results.* a) The results presented in Table 6 show exactly the same phenomenon for guinea-pigs as the ones described above for white rats, namely that the hypertrophy of the adrenals is significantly smaller in the group receiving more ascorbic acid. In fact, the group receiving 25 mgm. daily (per os) of ascorbic acid shows an adrenal enlargement of 9.1% which is statistically insignificant while the adrenal weight of the other group which received only 2 mgm. a day of Vitamin C is 28.6% greater in the cold room than at room temperature, and the result is highly significant ($t = 3.81$). The difference between the adrenal weights of each group is very small and not significant at room temperature, but significant (16.2%; $t = 2.62$ in the cold room.

The average ascorbic acid concentration of the adrenals is also shown. It is—as expected—larger in group II than in group IX; besides, if one compares the concentrations for ascorbic acid for the same group at room temperature and in the cold room respectively, one finds that for group II, more ascorbic acid is retained in the adrenals of the subjects exposed to cold: the difference is significant and is in line with our previous results (Dugal and Thérien, 1947). The difference for group IX, in the same conditions, is not significant.

b) One other experiment is summarized in Table 7: the groups

TABLE 7. INFLUENCE OF ASCORBIC ACID ON THE ADRENAL WEIGHT OF GUINEA-PIGS EXPOSED TO 2°C FOR 40 DAYS

	GROUP VI				GROUP IX				Difference in relative adrenal weight	t
	Number of animals	Body weight		Adrenal wt.	Number of animals	Body weight		Adrenal wt.		
		Initial	Final	Body wt. mgm./gm.		Initial	Final	Body wt. mgm./gm.		
Cold Room	24	437.4	506.8	0.6489	25	425.3	467.4	0.7628	17.5%	*2.32*
Room Temperature	25	449.1	654.8	0.4760	23	457.2	659.0	0.4549	4.6%	0.92

used received respectively 10 mgm. (Group VI) and 2 mgm. (Group IX) a day of ascorbic acid by mouth, the experiment lasted 40 days instead of 90, and the temperature was maintained constantly at 2°C throughout the experiment. The results presented are for adrenal weight as related to the body weight: this seemed to be the only logical procedure to follow, since all groups gained weight—even in the cold room—but the gain in weight was highly unequal for the different groups (in the cold room, the group receiving more ascorbic acid gained more weight).

Here again, it is obvious that ascorbic acid has an influence on adrenal weight at low temperature (the more ascorbic acid being taken, the smaller being the adrenal weight), but not at room temperature.

c) Oedema of the penis, one of the symptoms of non adaptation to cold (Leblond and Dugal, 1943) occurs more frequently in groups of guinea pigs receiving less ascorbic acid (ratio = 6:1 for the two experiments).

SUMMARY AND CONCLUSIONS

The typical enlargement of the adrenals under the influence of stress, observed many times before by other workers, and by ourselves for our controls in this actual series of experiments where cold was the damaging agent, *is completely prevented,* in rats and guinea-pigs exposed to cold if *they receive large doses of ascorbic acid.*

Surprising as it may seem, it is the very animals which have the smaller adrenals (those which receive the large doses of ascorbic acid) which are—and by far—the more resistant to cold.

Those experiments show that ascorbic acid seems to play a com-

pensatory role somewhat similar to the one of adrenal cortical hormones. The normal hypertrophy is prevented—although there is no atrophy—but, at the same time, the resistance to cold is increased. All those results, bringing new fundamental facts about the beneficial physiological effects of ascorbic acid in the cold, confirm our previous findings on the role of ascorbic acid for resistance and acclimatization to cold.

It is the first time, to our knowledge, that a substance other than a hormone, a substance which is *called* a Vitamin, is reported to prevent entirely, at least in the conditions that we have been using, the hypertrophy of the adrenals in a case of stress.

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THE ACTION OF TESTOSTERONE ON THE ADRENAL CORTEX OF THE HYPOPHYSECTOMIZED, PREPUBERALLY CASTRATED MALE MOUSE[1]

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THE ADRENAL cortex of the adult male and the parous female mouse consists of a connective tissue capsule surrounding the whole gland, an outer zona glomerulosa, a zona fasciculata and generally but not invariably a zona reticularis and a medullary connective tissue capsule. In young mice and in virgin females a further zone is recognizable, namely, the X zone and this shows variations with sex and age. In those mice with an X zone a medullary connective tissue capsule is not present and a zona reticularis is not well-developed or is absent (Waring, 1935; Jones, 1948a). For convenience of description the zona glomerulosa and the zona fasciculata (and the zona reticularis) are together termed the "permanent" cortex. In contradistinction to this term the X zone has been referred to as the "transitory" cortex (Whitehead, 1933) but the former term will be employed in this paper as it is now the name in common usage.

The variations of the X zone in the female mouse adrenal are not dealt with in this paper. In the male, however, the X zone disappears regularly at maturity, the degeneration usually being completed by 40 days (Howard, 1927; Deanesly, 1928; Whitehead, 1933; Waring, 1935 and others). Castration of the immature male mouse, preferably at about 21–25 days of age allows the X zone to remain and to increase in size. Such prepuberally castrated male mice have been studied by Deanesly and Parkes (1937), who showed that injections of androgens caused the disappearance of the X zone so that the adrenals then assumed the histological appearance characteristic of those of normal adult mice. Suppression of the X zone by testosterone in both male and female mice has also been shown (see Howard, 1940, for references). It is unknown whether or not this destructive action of androgens is a direct one on the X zone or mediated through the pituitary. The results of an experiment designed to elucidate this problem by the use of hypophysectomized, prepuberally castrated male mice

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are given here. Included in this paper are also the results of short term treatment with testosterone on the permanent cortex of the adrenals of the mice in the different experimental categories.

MATERIAL AND METHODS

Male mice of the "Swiss albino" strain were used. Twenty-eight were castrated at 21 days of age and left for 12 days (during which time the adrenal X zone persists and increases in size). At 33 days of age the mice were divided into the four groups delineated below. All the mice were killed at 42 days of age.

I. Six untreated castrated mice.
II. Six castrated mice injected subcutaneously once daily with 1 mg. of testosterone propionate in sesame oil.[3] The injections were started at 34 days of age, repeated for seven days, the animals being killed 24 hours after the last injection.
III. Six castrated mice were hypophysectomized and killed nine days later.
IV. Ten castrated mice were hypophysectomized. Subcutaneous injections, once daily, with 1 mg. of testosterone propionate in sesame oil[3] were given for 7 days, starting 24 hours after operation. The animals were killed 24 hours after the last injection.

The average of the body and the seminal vesicle weights of these four groups of mice are given in Table 1. The testosterone treated animals were livelier

TABLE 1. AVERAGE BODY AND SEMINAL VESICLE WEIGHT OF THE FOUR GROUPS OF MICE, AT DEATH

Group	Number in Group	Treatment*	Body Weight in gms.	Weight of seminal vesicles in mgs.
I	6	Castrated	18.8	2.4
II	6	Castrated; testosterone injected	21.7	45.1
III	6	Castrated; hypophysectomized	13.8	2.2
IV	10	Castrated; hypophysectomized, testosterone injected	15.8	28.5

* For details, see text.

and "sleeker" than the controls. However, the actual body weight differences between Groups I and II and between Groups III and IV are of doubtful significance. Seminal vesicle weights in Groups II and IV as compared to those of Groups I and III indicate that the testosterone was biologically active.

The operation of hypophysectomy was performed by the parapharyngeal approach. Confirmation of the completeness of hypophysectomy was made in the case of ten animals (5 from Group III and 5 from Group IV) by exami-

[3] "Perandren," Ciba Pharmaceutical Products, Inc., supplied through the courtesy of Dr. Houghton.

nation of sections made of the appropriate area. In the remainder, the success of the operation was judged by examination of the sella under the binocular microscope. It was found that this macroscopic examination is a complete confirmation once the individual technique is established. Hypophysectomy is here interpreted as the absence of the anterior and intermediate lobes (see Newton and Richardson, 1940, for discussion).

The mice, after castration and until the termination of the experiment, were kept on a high caloric synthetic diet (Shaw, 1947, gives the composition). It has been found that the chance of survival of hypophysectomized rats is greatly enhanced by the use of high caloric diets (Shaw and Greep, unpublished), and the results with mice used in this experiment and in other series confirm this (Jones, 1948b and unpublished).

The mice were killed with chloroform. The right adrenal was fixed in Bouin's fluid, embedded in paraffin, cut at 7μ and stained with Harris' haemotoxylin and eosin, or Heidenhain's Azan. The left adrenal was fixed in 10% formalin for at least 48 hours, washed in running water for an hour and sectioned on the freezing microtome at 15μ. Six sections from each gland were taken and treated as follows: (1) stained with Sudan black; (2) stained with Sudan IV; (3) stained with Sudan IV and counterstained with haemotoxylin; (4) treated with the Schiff reagent; (5) mounted unstained in glycerine and viewed with the polarizing and with the fluorescent microscopes; and (6) left in acetone at room temperature for half an hour and used as a control section for (5). These methods used on the left adrenal are discussed by Dempsey and Wislocki (1946) and have been employed extensively (see Deane and Greep, 1946; Greep and Deane, 1947; Dempsey, Bunting and Wislocki, 1947). In this paper, however, substances which react positively for all these tests are not regarded as necessarily indicating the presence of ketosteroids. Nevertheless criticism (Albert and Leblond, 1946; Yoffey and Baxter, 1947) which denies this specificity overlooks the extreme usefulness of the methods. The minimum evaluation of the histochemical tests employed here is that Sudan stains expose lipids, the Schiff reagent reacts with the aldehyde group and perhaps identifies in tissue sections the "plasmalogens" (Thannhauser and Schmitt, 1946), that examination with the polarizing microscope reveals anisotropic substances appearing after formalin fixation and that with the fluorescent microscope the presence and distribution of fluorescent substances are shown up in the adrenal cortex, and that these substances are acetone soluble.

Nevertheless, irrespective of any claim for specificity for any type of chemical compound, it is probable that the substances these tests reveal are fairly directly related to and fluctuate in accordance with the hormone (or hormones) so abundantly produced by the normal gland. Furthermore the reactivity with these tests is dependent on the endocrine status of the gland at the time and can be correlated with the state of activity of the adrenal cortex. For instance, good pointers to secretory state are given by consideration of the size and distribution of sudanophilic droplets within the cells, the predominance of the type of birefringence, whether coarse or fine, the intensity of the color after the Schiff reagent, and the particular areas which contain autofluorescent material. It is to be expected that tissues positive to some of these tests are scattered throughout the body. For example, any tissue with a molecular orientation is double refracting, fats in general are in

the class "lipids" and are sudanophilic, and Schiff positive substances are widespread throughout the body. However, the significant fact must not be overlooked that only the known steroid producing organs in their normal condition give positive results with all five of these tests. In brief, as regards the adrenal gland it is certain that these tests expose cortical changes in a more sensitive manner than the routine methods. Furthermore, these changes can be interpreted to throw light on the state of its activity.

RESULTS

1. The adrenal of the untreated prepuberally castrated male shows a well-developed X zone (Plate I, fig. 1). In the haemotoxylin and eosin preparations the zone is compact with the cytoplasm of the cells densely eosinophilic, the nuclei large and basophilic. The zone is unmistakable and has been described frequently after routine preparations as has the permanent cortex (Howard, 1927; Deanesly, 1928; Whitehead, 1933). After the lipid histochemical methods, the X zone is not sudanophilic, not Schiff-positive, contains no birefringent material (Plate II, figs. 5 and 9; Plate III, fig. 13) and is not autofluoresecent. The zona glomerulosa and zona fasciculata are positive for these tests; the Sudan and the Schiff reagents give strong colors (figs. 5 and 9) the reaction being within the droplets and autofluorescence being shown by both zones. The birefringent particles are both coarse and fine, scattered throughout the two zones (fig. 13). The acetone extracted control section is not autofluorescent or birefringent.

2. In the adrenal of the prepuberally castrated male injected with testosterone the X zone has disappeared and a medullary connective tissue capsule has formed (Plate I, fig. 2). The adrenal after the histochemical methods gives much the same picture as for the control (1 above). The Sudan and Schiff reactions, however, give less strong colors and there are fewer fine birefringent particles scattered in the cortex (Plate II, figs. 6 and 10; Plate III, fig. 14). A few big sudanophilic droplets lie in the juxtamedullary cells (fig. 6); these are Schiff

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Description of Plates

Plates I, II and III are section of adrenals from male mice, castrated at 21 days of age and killed at 42 days of age. Other experimental treatment in the different categories is indicated in each figure legend, the precise details being given in the text.

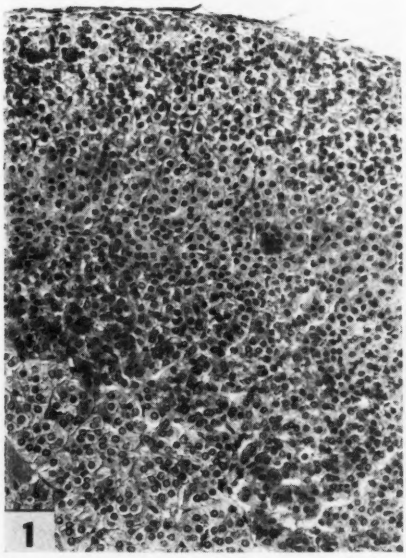
Plate I. Figs. 1 and 3 Harris' haemotoxylin and eosin; figs. 2 and 4 Heidenhain's Azan. ×190.

1. Castrated mouse. Juxtamedullary X zone well-developed.

2. Castrated mouse, injected with 1 mg. testosterone propionate daily for 7 days. The X zone has disappeared and a medullary connective tissue capsule formed.

3. Castrated mouse, hypophysectomized for 9 days. The X zone has condensed, the nuclei pycnotic; the zona fasciculata shows atrophy, the zona glomerulosa is little affected by the operation.

4. Castrated mouse, hypophysectomized for 9 days, injected with 1 mg. per day of testosterone propionate for 7 days. The X zone has disappeared, a medullary connective tissue capsule has formed; the zona fasciculata is atrophic, the zona glomerulosa is little affected.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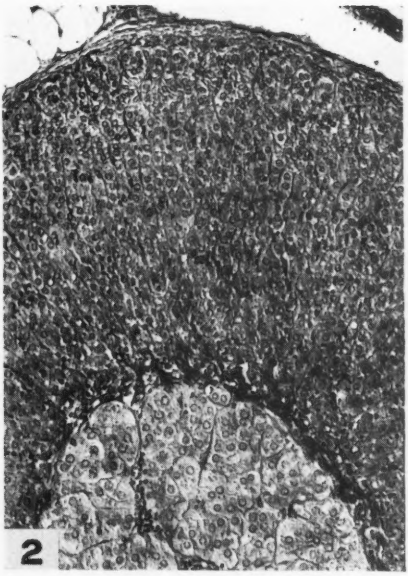
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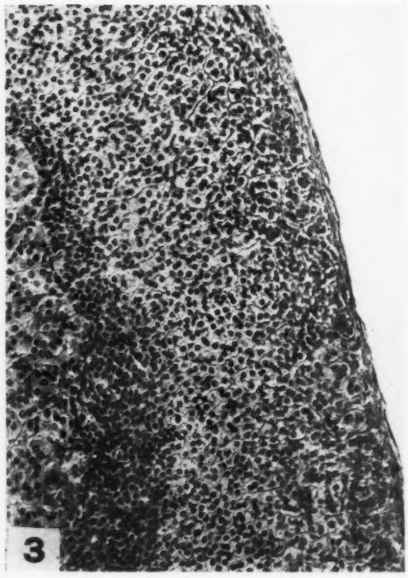
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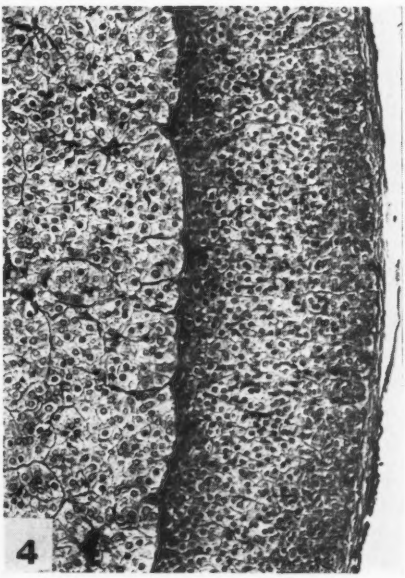
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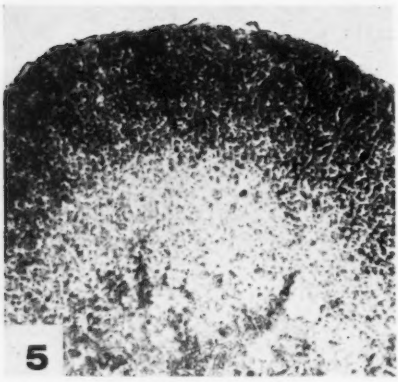
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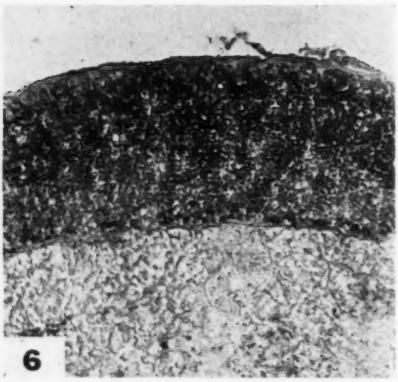
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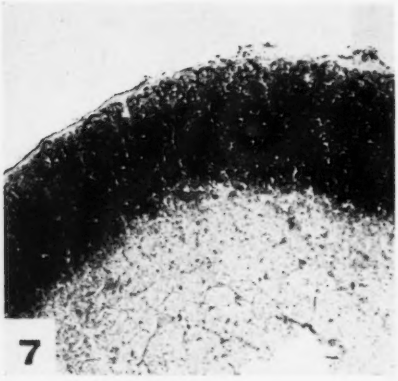
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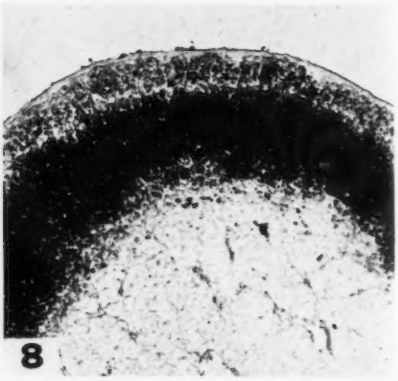
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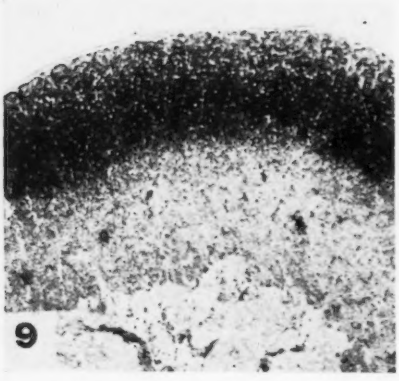
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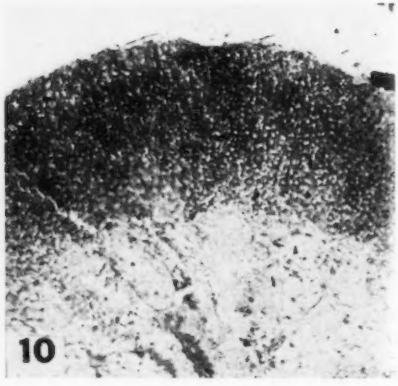
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negative (fig. 10) and do not contain birefringent particles (fig.14). The "permanent" cortex is autoflourescent. The acetone extracted control section is not autofluorescent or birefringent.

3. In the adrenal of the hypophysectomized, prepuberally castrated male, after the routine preparation, the zona fasciculata shows the changes characteristically consequent on this operation, with shrinkage of cell cytoplasm, and the pycnosis of nuclei. The zona glomerulosa shows little or no change. The X zone is still distinguishable; it is less eosinophilic, somewhat condensed, with the nuclei pycnotic (Plate I, fig. 3). After the histochemical methods, the sudanophilic droplets in the zona glomerulosa are coarser and less numerous than in the controls (Plate II, fig. 7), they are less Schiff-positive (Plate III, fig. 11), less autofluorescent, and the birefringent particles are coarse and few in number (Plate III, fig. 15). The zona fasciculata is intensely sudanophilic, with the droplets large and tending to coalesce, the color after Schiff is intense; the birefringent particles are coarse for the most part and show a tendency to clump (figs. 7, 11 and 15); autofluorescence is slight to absent. The acetone extracted control section is not autofluorescent or birefringent.

4. In the adrenal of the hypophysectomized prepuberally castrated male injected with testosterone, the X zone has disappeared and a medullary connective tissue capsule formed (Plate I, fig. 4). The routine preparation show the zona glomerulosa and the zona fasciculata in the condition noted for 3 above—the uninjected hypophysectomized animal. The histochemical tests on the left adrenal give constant differences, however. The zona glomerulosa has fewer sudanophilic droplets (Plate II, fig. 8); it is only faintly Schiff-positive or not at all (Plate III, fig. 12); to find birefringent particles in it is rare and these when present are coarse (Plate III, fig. 16); it is not autofluorescent. The zona fasciculata is intensely sudanophilic (fig.

PLATE II. Magnification ×90. Adrenal sections at 15μ made on the freezing microtome.

5. Castrated mouse, adrenal after Sudan IV. The zona glomerulosa and zona fasciculata have taken the Sudan stain, the X zone lying between the latter zone and the medulla (at the bottom of the photograph) has not.

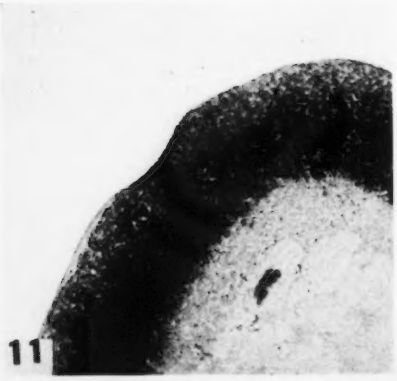
6. Castrated mouse injected with testosterone for 7 days, adrenal after Sudan IV. The Sudan droplets are somewhat sparser and more irregularly distributed in the zona glomerulosa and zona fasciculata. The X zone has been destroyed. A few small very sudanophilic areas lie against the medullary connective tissue capsule.

7. Castrated mouse hypophysectomized for 9 days, adrenal after Sudan IV. The zona fasciculata is densely sudanophilic with coarse droplets; there is rather less sudanophilic material in the zona glomerulosa but the amount present is densely stained.

8. Castrated mouse hypophysectomized for 9 days, injected with testosterone. Adrenal after Sudan IV. The zona fasciculata is densely sudanophilic, the droplets are coarse. The zona glomerulosa is sparsely sudanophilic and can be clearly demarcated from the zona fasciculata.

9. Castrated mouse, adrenal after the Schiff reagent. The zona glomerulosa and zona fasciculata are Schiff positive, the X zone is Schiff negative.

10. Castrated mouse injected with testosterone, adrenal after the Schiff reagent. Both the zona glomerulosa and zona fasciculata are Schiff positive, the color is less intense than that of the control, fig. 9.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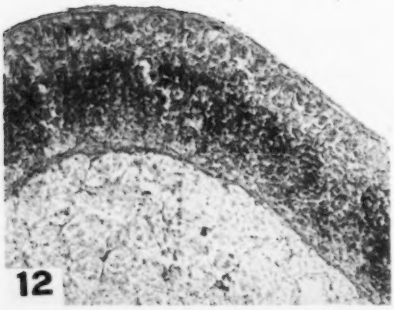
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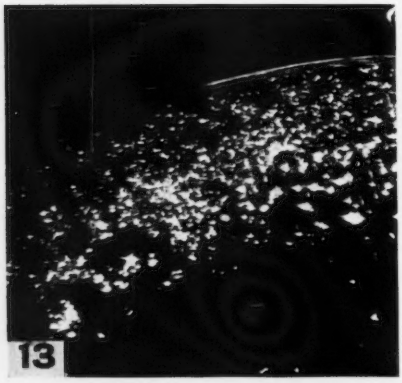
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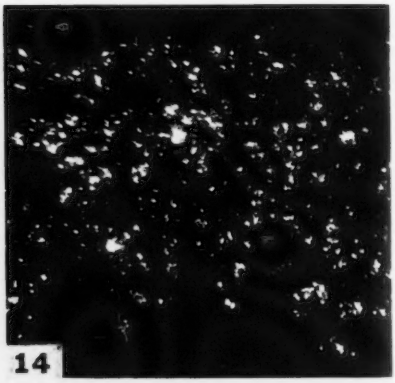
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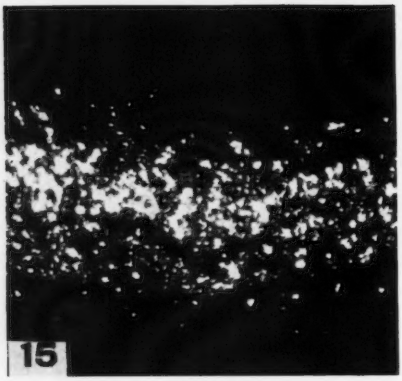
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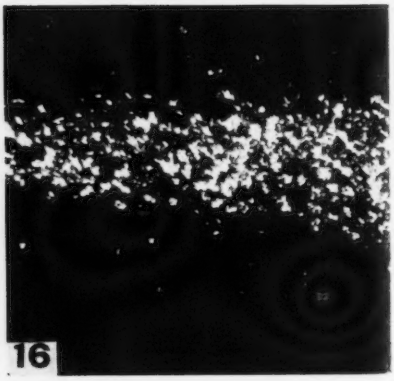
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8), weakly Schiff positive (fig. 12), and full of coarse birefringent particles, largely clumped together (fig. 16). It is faintly autofluorescent or not at all. The acetone extracted control section is not autofluorescent or birefringent.

DISCUSSION

The persistent adrenal X zone of the prepuberally castrated male mouse is rapidly destroyed by the injection of testosterone and a medullary connective tissue capsule forms. In the hypophysectomized prepuberally castrated male the X zone becomes atrophic but persists as a condensed layer (X zone remnants can still be recognized up to 40 days after operation. (Jones, 1948b and unpublished). This X zone is swept away by exogenous testosterone and a medullary connective tissue capsule appears. It would seem that the action of testosterone on the X zone is a direct one, in the absence of the X zone maintaining principle of the pituitary. This latter has been shown (Jones, 1948c) to be a pituitary gonadotropin, possibly 'LH'. We envisage the mechanism of X zona destruction in the normal male mouse then to be as follows—the androgens produced concomitant with maturity reduce the secretion of pituitary 'LH' (Greep, Van Dyke and Chow, 1942) until a balance is formed and at the same time the androgens act directly on the X zone to destroy it, this destructive action being aided perhaps by the lessening secretion of 'LH'—the X zone maintaining factor.

The function of the X zone is not known. It has frequently been referred to as the "androgenic" zone and assigned the function of androgen secretion. There is no experimental evidence for this view (Gersh and Grollman, 1939a and b; McPhail and Read, 1942; and Howard, 1946). It is significant that the well developed X zone is

PLATE III. Figs. 11, 12, ×90. Figs. 13 to 16, ×120. Adrenal sections at 15μ made on the freezing microtome.

11. Castrated hypophysectomized mouse, adrenal after Schiff reaction. Positive reaction in permanent cortex, the X zone negative. The zona glomerulosa shows less color than the zona fasciculata, which takes the stain intensely.

12. Castrated hypophysectomized mouse, injected with testosterone, adrenal after Schiff reaction. Little reaction in the zona glomerulosa, positive in the zona fasciculata but weakly so. No X zone.

13. Castrated mouse. Plain section of adrenal mounted in glycerine taken with the polarizing microscope. The permanent cortex has scattered in it a mixture of coarse and fine birefringent particles. The X zone does not show as it contains no birefringent material.

14. Castrated mouse injected with testosterone. Plain section of adrenal mounted in glycerine taken with the polarizing microscope. The cortex contains a mixture of fine and coarse birefringent particles but these are sparse. The apparent wider cortex is not significant, merely being the chosen area.

15. Castrated hypophysectomized mouse. Plain section of adrenal mounted in glycerine taken with a polarizing microscope. The zona glomerulosa shows a few scattered, coarse birefringent particles. The zona fasciculata shows a clumping of this material.

16. Castrated hypophysectomized mouse injected with testosterone. Plain section of adrenal mounted in glycerine taken with a polarizing microscope. Hardly any birefringence in the zona glomerulosa, the zona fasciculata with clumps of coarsely birefringent particles.

negative for those tests for which the known steroid-producing tissues of the body are positive. The tests when positive show up in the adrenal cortex substances in addition to the steroid hormones. When these tests are negative, it seems likely that neither these substances nor the steroid hormones are present. Alternatives to this interpretation are first that the hypothetical steroid hormone of the X zone is produced and passed into the blood stream so quickly that it is not "caught" by fixation; or second that this hypothetical hormone is not elaborated in the same way as the steroids are thought to be in the permanent adrenal cortex. If either of these alternatives were true, then the fact that the X zone is negative to the tests used would not be conclusive. There is no evidence available which would decide the issue.

Short term injection of testosterone propionate had only a slight effect on the permanent cortex of the prepuberally castrated mouse. Based on the birefringence, which was lessoned as compared with the controls and for the most part coarse in nature, the indication is that the secretory activity of the cortex has diminished. For, in general, heightened activity is reflected in increased fine birefringence and diminished activity by increased coarse birefringence (Weaver and Nelson, 1943; Deane, Shaw and Greep, 1948). The changes in the results from the other tests, as compared with the normals, are not definite enough to be conclusive. Testosterone injections have, however a marked effect on the adrenal as judged by histochemical tests when these animals are hypophysectomized. In this case the changes consequent on hypophysectomy are hastened and the adrenals of the injected mice 9 days after operation have the appearance of those from mice about 15 days or so after operation (from another series Jones, 1948b and unpublished). The zona glomerulosa has a lessened content of substances positive for the histochemical tests as compared to the untreated hypophysectomized animals, although in the routine preparation the zone is hardly changed in the adrenals of mice from any of the experimental categories. Further experimentation is required before these results in the zona glomerulosa can be interpreted. In the zona fasciculata there is much coalescence of the big sudanophilic droplets, the Schiff reaction is weak, and the birefringent particles are all coarse and clumped together. It is clear that the atrophy of the zona fasciculata of the hypophysectomized animal has been increased by testosterone injection. It appears then that testosterone can have a direct atrophic effect on the permanent cortex in the absence of the pituitary.

The writer is unaware of any other observations on the results of androgen injections into hypophysectomized mice. In the rat Leonard (1944) reported that testosterone injected into hypophysectomized animals had an "adrenocorticotrophin-like" effect on the adrenals in that the adrenal atrophy normally consequent on the operation did

not occur and that the glands were heavier than those of the untreated hypophysectomized controls. The results of Leonard are surprising especially in view of the fact that exogenous androgens do not cause hypertrophy of the adrenals in either the rat or the mouse, indeed atrophy is the usual consequence (see Selye, 1939; Parkes, 1945). Also adrenal "castration hypertrophy" in the rat is removed by androgen injections (Hall and Korenchevsky, 1938). The earlier report on the results of testosterone injection into the hypophysectomized rat by Cutuly *et al.* (1938) is more in accord with the results with the mouse given here. They noted that the adrenals of hypophysectomized rats after androgen treatment had histologically the typical shrunken cortices characteristically following hypophysectomy although 5 out of 12 adrenals were heavier than those of hypophysectomized controls. It is interesting to note that in another species, the ground squirrel, Zalesky *et al.*, (1941), found that testosterone injections had no effect on the atrophy or weights of the adrenals of hypophysectomized animals. On the whole it seems clearly established that testosterone can have a direct effect on the "permanent" cortex of the adrenal (that is without pituitary mediation) and that this action in the mouse is in the direction of atrophy. The physiological significance, however, of the action of androgens on the adrenal of either the intact or the operated animal is unknown.

SUMMARY

The adrenal X zone persists in the prepuberally castrated male mouse and the zone is destroyed by testosterone injections. Likewise, the X zone persists, although atrophic, in the adrenal of the hypophysectomized, prepuberally castrated mouse and it is swept away by testosterone injections. The action of the testosterone on the X zone of the normal adrenal seems, therefore, to be a direct one.

The X zone of the adrenal is negative to those histochemical tests for which the permanent adrenal cortex and other steroid-producing organs are characteristically positive. This is in agreement with the view that a steroid-type hormone is not secreted by the X zone.

Injections of testosterone into hypophysectomized animal hastens the changes, as judged histochemically, in the zona glomerulosa and zona fasciculata of the adrenal consequent upon the operation.

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CONCENTRATION OF ADRENOCORTICOTROPHIC HORMONE IN RAT, PORCINE AND HUMAN PITUITARY TISSUE[1]

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THE DEVELOPMENT of an accurate and sensitive method for the assay of adrenocorticotrophic hormone (ACTH), namely, the adrenal ascorbic acid-depletion method of Sayers, Sayers and Woodbury (1948), has made possible the quantitative determintaion of the concentration of this trophin in pituitary tissue. The adenohypophyses of three species—rat, hog and human—have been analyzed in this regard.

METHODS AND MATERIALS

Technic for Quantitative Extraction of ACTH from Pituitary Tissue. During the initial phases of this study, extracts of anterior pituitary were prepared by grinding or homogenizing the fresh tissue in cold 0.9 per cent NaCl solution made alkaline to 0.01 N with NaOH. The technic is similar to those described in the literature for the measurement of pituitary gonadotrophic hormone potency. When pituitaries from a large number of control groups of rats were extracted by this method and found to vary widely in ACTH content, the technic of grinding or homogenizing fresh tissue was abandoned as unsatisfactory. A simple method was subsequently devised for the quantitative extraction of ACTH from pituitaries. The glands were first frozen, then lyophilized, and the dry tissue ground to a homogeneous powder. Extracts of this material were very potent and uniform in ACTH activity. Examination of Table 2 shows that extracts prepared from tissue which was first homogenized and then lyophilized were at best only one-fifth as potent (antilog of M equal to 0.19) as extracts from pituitaries lyophilized before being ground (Table 2).

The routine procedure now employed in this laboratory for extracting ACTH from pituitaries is as follows: Approximately three-quarters of a milligram of dry, lyophilized pituitary are weighed on a micro-analytical balance to the nearest 0.005 mg. Larger samples may be employed according to the total weight of tissue available. The tissue is extracted at room temperature successively with three approximately equal portions of 0.9 per cent NACl solution made alkaline to 0.01 N with NaOH. The residue is centrifuged down after each extraction and the supernatant portions are combined. An

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example of a typical extraction follows: the weight of dry, donor pituitary tissue was 0.725 mg.; the total volume of extraction fluid was 0.5 ml. per 0.04 mg. of tissue (the largest dose employed per 100 gm. of recipient rat), and was equal in this instance to 9.1 ml.; the tissue was extracted successively with 3.1, 3.0 and 3.0 ml. portions of the extraction fluid; two dilutions were prepared from the combined extracts by the addition of 0.9 per cent NaCl solution so that the concentration of tissue per 0.5 ml. was 0.02 mg. and 0.01 mg. respectively. The recipient rats were injected with either 0.5 ml. of the original extract or 0.5 ml. of one of the dilutions thereof, per 100 gm. of body weight.

Extraction of ACTH from the pituitary appears to be complete. Whereas the thrice-extracted material prepared as above gave an appreciable adrenal cortical response in recipient rats at a dose level equivalent to 0.005 mg. per 100 gm., a fourth extraction of the residue failed to elicit a response at a dose level equivalent to 1.0 mg. per 100 gm.

Source of Pituitaries. Rat pituitaries were obtained as follows: Male Sprague-Dawley rats weighing 250 to 350 gm. were fasted 24 hours, anesthetized with sodium pentobarbital and exsanguinated by cutting the abdominal aorta; the pituitaries were removed within a minute after death. The anterior lobes, freed from posterior tissue, were frozen and lyophilized. The assays were conducted on pools of rat pituitaries.

Pituitaries were removed from the hogs not later than 10 minutes after death. Six porcine anterior lobes were pooled, frozen, lyophilized and ground to a homogeneous powder.

Anterior lobes of three human pituitaries were obtained through the courtesy of Dr. C. McNeil, Holy Cross Hospital, Salt Lake City. They were frozen within two hours after removal and lyophilization was started immediately after freezing. Human pituitary #1 was from a 76-year old male who died approximately 24 hours after a myocardial infarction. The pituitary was removed five hours after death. Human pituitary #2 was from a 58-year old male with a bronchogenic carcinoma; death was due to peritonitis from a perforated peptic ulcer. The pituitary was removed eight hours after death. A cyst of the pituitary was not included in the anterior lobe tissue taken for ACTH analysis. Human pituitary #3 was from a full-term, stillborn infant with erythroblastosis fetalis. The pituitary was removed approximately five hours after death.

Reference Standards. Two reference standards of ACTH activity were employed. Preparation 62AA was prepared from porcine pituitary tissue according to the method of Sayers, White and Long (1943). This protein behaved as a single component in the Tiselius apparatus and in the ultracentrifuge; it was free of lactogenic, growth, gonadotrophic and thyrotrophic activities. Preparation La-1-A, isolated from porcine glands, was supplied by Dr. John Mote of the Armour Laboratories. The data in Table 2 indicate that La-1-A is slightly more potent than 62AA. The slopes of the log dose-response curves of these two preparations are not significantly different.

Assay Procedure. Twenty-four hours after hypophysectomy the recipient assay rat was anesthetized with sodium pentobarbital and the left adrenal removed and prepared for the analysis of ascorbic acid. The extract to be assayed was injected via the tail vein, 0.5 ml. per 100 gm. body weight, and one hour later the right adrenal was removed and prepared for ascorbic acid

TABLE 1. ASSAY OF HUMAN PITUITARY #1 AGAINST LA-1-A BY THE DIFFERENCE METHOD

Dose (micrograms per 100 gm. body weight)		Concentration of adrenal ascorbic acid (mg. per 100 gm. of tissue)					
		Human Pituitary #1			La-1-A		
Human Pituitary #1	La-1-A	Left Adrenal	Right Adrenal	Difference	Left Adrenal	Right Adrenal	Difference
8	2	390	229	161	350	218	132
8	2	425	240	185	355	214	141
–	2	—	—	—	463	264	199
4	1	359	236	123	412	272	140
4	1	415	315	100	427	300	127
4	1	439	383	56	350	236	114
4	–	307	237	70	—	—	—
2	0.5	337	280	57	416	367	49
2	0.5	397	365	32	481	392	89
2	0.5	457	405	52	384	306	78

	Human Pituitary #1	La-1-A
$b =$	185.6	141.8
$s =$	25.66	24.32
$\lambda = s/b =$	0.138	0.172

$M = 1.2719$; ratio of potencies Human pituitary #1/La-1-A = 0.187

$S_M = \pm 0.0242$; limits of error at p = 0.67 are 95% to 106%

Range of potencies Human pituitary #1/La-1-A in 67 cases out of 100 = 0.177 – 0.198

analysis. The ACTH potency of the extract is proportional to the decrease in adrenal ascorbic acid as measured by the difference between the concentration of ascorbic acid in the left (control) and right adrenal. The details of the method have been presented elsewhere (Sayers, Sayers and Woodbury, 1948).

The tissue was extracted on the morning of the assay and the extracts and dilutions thereof were kept at 0°C until assayed. Unknown and standard preparations were compared on the same day except in the cases of human pituitaries #2 and #3. Doses of unknowns were selected so that the responses obtained closely approximated those of the standard. It was therefore necessary to establish the approximate potency of the unknown extracts by *preliminary* assays before proceeding to the *final* quantitative analyses. A sample protocol of such a final assay, presented in Table 1, represents the assay of human pituitary #1 against a purified preparation of ACTH (La-1-A, Armour) used as a standard.

RESULTS

In Table 2 are presented in condensed form the data from all final assays performed in this experiment; they were compiled in a manner similar to that for the data of Table 1. In no instance was there a significant difference between the slopes (b values) of the log dose-response curves of the substances compared. The probability, P, corresponding to Fisher's t, is either 0.2 or greater for each difference in the slopes.

The antilog of M in Table 2 gives the ratio of the potencies of unknown to standard. Of the three species examined, rat pituitary tissue (in terms of dry weight) has the lowest order of adrenocorticotrophic hormone potency; it is only 0.08 as potent as La-1-A. The hog and human pituitaries analyzed in this study are very potent; in terms of dry weight, they are one-fifth as potent as the purified standards.

Two pools of rat anterior lobes collected on different dates had the same potency of adrenocorticotrophic activity. The three human pituitaries, despite the marked differences in age and causes of death of the donors, were quite uniform in their content of ACTH.

DISCUSSION

The lyophilization technic reported here permits a quantitative extraction of ACTH from pituitary tissue. This fact together with the fact that the assay method is highly specific (Sayers, Sayers and Woodbury, 1948) suggests that the values presented above for ACTH concentration in pituitary tissue are very close to the true values.

The grinding or homogenizing of fresh tissue, a procedure routinely employed in the preparation of extracts of pituitary for the assay of gonadotrophins, yields extracts which are at best only one-

TABLE 2. CONCENTRATION OF ACTH IN PITUITARY TISSUE

Pituitaries compared	No. recipient rats	b	P	s	λ	M	Antilog of M	S_M	Antilog (M $-S_M$)–Antilog (M $+S_M$)
Rat[1] (homogenized) (16)[2]	24	80	0.2	20.4	0.255	$\bar{1}$.289	0.19	±0.113	0.15–0.25
Rat[1] (lyophilized) (10)	24	113		25.4	0.224				
62AA[1,3]	12	169	0.3	20.0	0.119	$\bar{1}$.926	0.84	±0.033	0.78–0.91
La-1-A[1,3]	14	196		30.3	0.155				
Rat[1] (13)	19	152	0.8	21.9	0.145	$\bar{2}$.911	0.08	±0.043	0.07–0.09
La-1-A[1]	19	158		17.8	0.112				
Rat[1] (19)	12	149	0.3	26.3	0.177	$\bar{1}$.995	0.99	±0.072	0.84–1.17
Rat[1] (10)	24	113		25.4	0.224				
Porcine (6)	10	112	0.3	11.4	0.102	$\bar{1}$.299	0.20	±0.084	0.16–0.24
La-1-A	10	79		22.3	0.282				
Human #1	9	186	0.4	25.7	0.138	$\bar{1}$.272	0.19	±0.024	0.18–0.20
La-1-A	9	142		24.3	0.172				
Human #2	10	124	0.7	29.0	0.234	$\bar{1}$.309	0.20	±0.093	0.16–0.25
La-1-A	9	142		24.3	0.172				
Human #3	6	156	0.7	19.5	0.125	$\bar{1}$.381	0.24	±0.081	0.20–0.29
La-1-A	9	142		24.3	0.172				

b = slope of the log dose-response curve; P = probability factor measuring the significance of the difference between the slopes; s = standard deviation of all of the individual responses about this curve (a straight line fitted by the method of least squares); λ = s/b and is a measure of the accuracy of the assay method; M = logarithm of the ratio of the potencies; S_M = standard error of M; antilog (M $-S_M$)–antilog (M $+S_M$) is the range of estimate for one std. error.

[1] Several assays combined for statistical analysis.

[2] No. animals in donor groups in parentheses.

[3] La-1-A and 62AA are purified preparations of ACTH isolated from porcine pituitaries.

fifth as potent in adrenocorticotrophic activity as those obtained by the lyophilization technic. It would be of interest to compare the gonadotrophic potency of extracts of pituitary tissue prepared by "standard" methods with that of extracts prepared by the technic developed in this study.

Lyophilized pituitaries may be stored in a desiccator until such time as it is convenient to assay the ACTH activity. There has been no indication that a decrease in potency takes place over a period of at least one year when the tissue is stored in this manner.

The fact that there is no significant difference between the slopes of the log dose-response curves of purified porcine ACTH standards and of crude extracts of rat, porcine and human pituitaries suggests that the biological characteristics of adrenocorticotrophin in the several species and in crude and purified preparations are similar. Li *et al.* (1943) and Sayers *et al.* (1943) isolated from sheep and hog pituitaries, respectively, biologically pure and chemically homogeneous proteins having high adrenocorticotrophic activity. Both of these proteins had molecular weights of 20,000. However, reports have appeared in the literature to the effect that the hormone activity is dialyzable and ultrafiltrable (Tyslowitz, 1943; Crooke *et al.*, 1947). Li (1947), in a note lacking experimental details, claims that hydrolysis of ACTH yields active polypeptides with an average amino acid chain-length of seven. In this laboratory[2] it has been found that the adrenocorticotrophic activity of extracts of lyophilized pituitary tissue will not pass an ultrafiltration membrane whose pores are large enough to allow protamine to pass freely. Although it is fairly certain that the biological properties of isolated preparations of ACTH and of the hormone as it exists in pituitary tissue are identical, there is little certainty concerning the size of the protein (or of the moiety of protein) which carries the biological activity.

If it is assumed that the molecular weight of the hormone as it exists in the pituitary is 20,000, *i.e.*, the same as that of the standard 62AA, then one-fifth of the dry weight of porcine and human adenohypophyseal tissue consists of ACTH. The average dry weight of the anterior lobe of the adult hog is about 60 mg.; therefore this animal has a store of about 12 mg. of ACTH. The average dry weight of the anterior lobe of the adult human is about 100 mg.; therefore this species has a store of about 20 mg. of ACTH. Such large stores ensure a ready supply of adrenocorticotrophin in times of emergency. This is clearly illustrated by experimental data on the rate of discharge of ACTH from the pituitary during stress. Employing the adrenal ascorbic acid-depletion method for measuring rate of discharge of ACTH, Sayers and Sayers (1947) found that application of mild, short-acting forms of stress in rats resulted in a rate of discharge of

[2] Unpublished observations of John B. Richards, Thomas W. Burns and George Sayers.

ACTH equivalent to approximately one microgram of standard per hour. From the information in Table 2, the total ACTH in the pituitary gland of the rat can be calculated to be about 100 micrograms, a quantity far in excess of immediate needs of the animal when exposed to mild stress. However, following the application of severe stress, the content of ACTH in the rat pituitary may be reduced by one-half within a period of one hour (Sayers and Cheng, 1949). If this information is applicable to man, then a quantity of ACTH of the order of 10 mg. may be discharged from the adenohypophysis of the adult within a period of a few hours following a severe injury or acute metabolic disturbance.

SUMMARY

A method has been devised for the quantitative extraction of adrenocorticotrophic hormone from pituitary tissue. The fresh tissue is frozen, lyophilized and ground to a homogeneous powder. The powder is extracted with 0.9 per cent NaCl solution made alkaline to 0.01 N with NaOH.

The concentration of ACTH in pituitary tissue has been expressed in terms of a purified standard isolated from hog pituitaries. Rat, porcine and human adenohypophyseal tissues, in the form of dry powders, are eight, 20, and 21 per cent as potent as the standard, respectively. The biological characteristics of the hormone, as it exists in the purified standard and in the glands of the three species examined, are similar.

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THE PHYSIOLOGICAL ACTIVITIES OF THE STEREOISOMERS OF THYROXINE

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Most workers have assumed that D-thyroxine will have a lesser physiological activity than the naturally occuring L isomer, although there is as yet no quantitative agreement on their relative activities. In 1928 Harington prepared the D & L isomers and their activities were measured by Gaddum (1929–30) and Salter, Lerman and Means (1935). Gaddum measured the oxygen consumption of rats and found the L isomer to be more effective than the D isomer which had one third to two thirds the activity, depending on the dose given. Using metamorphosis of the tadpole as the criterion of activity, essentially the same results were obtained. Salter, Lerman and Means (1935) assayed the same materials in human myxoedema patients and found them to be equally active. Foster, Palmer and Leland (1936), measuring the carbon dioxide production in guinea pigs, found the DL form to be only one half as active as the L form, whence they inferred D-thyroxine to be inactive. Reineke and Turner (1943) and Reineke, Turner, Kohler, Hoover and Beezley (1945) made similar measurements. Reineke and Turner (1945) determined the relative activities of L- & DL-thyroxine in inhibiting the increase in thyroid weight of rats and chicks receiving thiouracil, and in accelerating metamorphosis of tadpoles. In all these comparisons the DL form was found to be half as active as the L form, supporting the view that the D isomer was inactive. Griesbach and Purves (1945) showed that the smallest amount of thyroxine necessary to prevent thyroxine deficiency changes in the pituitary was sharply defined and could be used as a measure of thyroxine requirement. Using this method, Griesbach, Kennedy and Purves (1947) compared three different samples of L-thyroxine with DL-thyroxine. All three samples of L-thyroxine showed equal activity corresponding to about 1.5 times that of DL-thyroxine. This would make D-thyroxine about one third as active as L-thyroxine. Pitt Rivers and Lerman (1947) prepared D-thyroxine and compared this material with the L isomer in human myxoedema patients, finding it to have between one eighth and one tenth the activity of L thyroxine.

These results are summarized in Table 1.

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TABLE 1. ACTIVITIES OF D & L THYROXINE AS REPORTED BY DIFFERENT WORKERS

Observer	Method	Forms compared	D:L Ratio found or calculated
Gaddum 1929/30	O_2 consumption of rat	D & L	0.66–0.33:1
	Metamorphosis of tadpole	D & L	0.8 –0.5 :1
Salter et al. 1935	Human myxoedema	D & L	1:1
Foster et al. 1936	O_2 consumption of guinea pig	DL & L	0:1 calc.
Reineke et al. 1943	CO_2 production in guinea pig	DL & L	0:1 calc.
Reineke et al. 1945	CO_2 production in guinea pig	DL & L	0:1 calc.
Reineke et al. 1945	Thyroid wt. of rat receiving thiouracil	DL & L	0:1 calc.
	Thyroid wt. of chick receiving thiouracil	DL & L	0:1 calc.
	Metamorphosis of tadpole	DL & L	0:1 calc.
Griesbach et al. 1947	Inhibition of pit. change in methylthiouracil rat	DL & L	0.3 :1 calc.
Pitt Rivers et al.	Human myxoedema	D & L	0.125–0.1 :1

We have now been able to make a direct comparison of D- & L-thyroxine, results of which are reported in this paper.

MATERIAL AND METHODS

The thyroxine samples used were: L-thyroxine prepared by oxidation of L-diiodo-tyrosine according to the method of Harington and Pitt Rivers (1945), being part of the material used in a previous experiment (Griesbach, Kennedy and Purves 1947): DL-thyroxine prepared by alkaline hydrolysis of thyroid: D-thyroxine prepared by oxidation of D-diiodo-tyrosine. This material was part of that prepared by Pitt-Rivers and Lerman. Sufficient material for testing in three rats only was available. The L & DL samples were twice recrystallized as the mono-sodium salt. Solutions were prepared by dissolving the weighed material in an excess of 0.1 N sodium carbonate and diluting with normal saline so that the final solutions contained 7.5 μg. DL-, 5 μg. L- and 10 μg. D-thyroxine respectively per millilitre. The strength of the solutions was confirmed by iodine analysis.

Young male albino rats of 50–70 gms. weight were used. They were placed on a strict vegetarian diet and methylthiouracil was given as an 0.025% solution in the drinking water. They were weighed twice weekly and the thyroxine dosage was adjusted to the body weight. The thyroxine was given subcutaneously once daily for seventeen to twenty days. The pituitaries were then examined as described by Griesbach and Purves (1943).

RESULTS

The smallest dose of DL-thyroxine which prevented the development of a pituitary basophilia was 2.3 μg. per 100 gms. per day. The

equivalent value of L-thyroxine was 1.5 μg. per 100 gms. per day. In the case of D-thyroxine 5 μg. per 100 gms. per day gave a pituitary basophil count of 9.6% and 4 μg. a count of 28.5%. The animal receiving 10 μg. per 100 gms. per day had a count of 4.1%. These pituitary basophils were small, filled with well stained granules, and there was a complete absence of activated basophils, a certain proportion of which is found in our control animals. The thyroid showed subnormal activity with flattened epithelium and dense colloid.

These results are summarized in Table 2.

TABLE 2. THE EFFECT OF GRADED DOSES OF THYROXINE STEREOISOMERS ON THE PITUITARY BASOPHIL PERCENTAGE OF METHYLTHIOURACIL TREATED RATS

Form given	No. of animals	Dose μg/100gm/day	Mean pituitary basophil % ±S.E.M.
—	20	—	30
DL	10	2.2	14.2±0.75
	10	2.3	8.4±0.92
	10	2.4	6.5±0.73
L	10	1.4	14.9±1.40
	10	1.5	6.8±0.98
	10	1.6	6.5±0.74
D	1	4.0	28.5
	1	5.0	9.6
	1	10.0	4.1
Animals without thyroxine and methylthiouracil			7–10

The ratio of activities D:L found by direct comparison is 0.30:1 and is also 0.30:1 as calculated from the L:DL ratio 2.3:1.5.

DISCUSSION

In attempting to evaluate the different results obtained for the activities of thyroxine stereoisomers, it should be remembered that in most cases different methods, species and materials have been used. In one case, however, the same materials have been compared in the same species: Reineke and Turner's L-thyroxine in the rat (Reineke and Turner, 1943 and Griesbach, Kennedy and Purves, 1947). The conflicting results obtained here must be attributed to the use of different methods. The thyroid weight method seems to be much less sensitive than the pituitary response, for the basophils show considerable change when the thyroxine supplied is varied by 0.1 μg. in the vicinity of the end point. This should be contrasted with the dose interval of 0.5 μg. used by Reineke and Turner (1945). In the guinea pig, the apparent inactivity of the D isomer is based on observations that approximately the same increase in metabolism was produced by doses of DL-thyroxine twice those of the L isomer. The errors inherent in the use of this relatively insensitive method are such that a

small activity for the D isomer could not be detected with certainty. The results of Salter, Lerman and Means (1935) and Pitt Rivers and Lerman (1947) obtained in human myxoedema patients, while lacking in quantitative agreement, definitely establish the fact that D-thyroxine is physiologically active in man. Our work has shown that the same holds true in the rat.

SUMMARY

The thyroxine requirement of the rat was found to be 2.3 μg. of DL-thyroxine per 100 gms. per day, 1.5 μg. of L-thyroxine and 5 μg. of D-thyroxine.

D-thyroxine has been shown to have biological activity in the rat equal to 0.3 times that of L-thyroxine.

This result has been obtained by direct comparison of the D & L isomers and also by calculation from the L:DL ratio.

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INFLUENCE OF THYROXINE ON PROTEIN METABOLISM

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IT IS CUSTOMARY to regard the thyroid hormone as a stimulator of catabolism, in particular of protein catabolism. However, the impaired growth of human cretins, of thyroidectomized rats (Evans, Simpson, and Pencharz 1939) and goats (Reineke, and Turner 1941) as well as the resumption of growth resulting from thyroid medication indicate that the influence of thyroid hormone on metabolic processes is more complex. Growth is, of necessity, associated with a prevalence of anabolic processes, especially of proteins. The experiments reported in this paper were undertaken in order to determine, (a) whether thyroxine induces nitrogen retention (protein anabolism), and (b) if so, under what conditions anabolic or catabolic effects would predominate.

MATERIAL AND METHODS

The rats used in most of the experiments were descendants of the Wistar Strain, bred by a local dealer; rats of the Sprague-Dawley strain were employed in some instances. Their weights varied between 200 and 250 grams. Thyroidectomy was performed by the usual anterior approach, and hypophysectomy by the parapharyngeal route. Experiments on operated animals were started three weeks following operation. However, in one experiment the animals were tube-fed constant amounts of diet prior to operation, on the day of operation, and in the post-operative period. In this experiment (Table 2) the post-operative period refers to days 14–21 following thyroidectomy.

All animals were tube-fed, using a number 10 french catheter. The "Mixed diet" described by Ingle *et al.* (Ingle, Ginther, and Nezamis 1943) was employed. Samples of the diet were frequently ashed and the N-content, determined by the Kjeldahl method, was found to be 16 mg. per ml. Each animal was gradually adapted to tube feeding, and the amount was fed which maintained weight during the control period. Thus the intake varied from one animal to another, but was kept rigidly constant for each rat during the control and experimental periods. Feedings were at 7:30 A.M. and 5 P.M., except for the hypophysectomized animals which were fed at 7 A.M., 3 P.M. and 10 P.M. All animals had free access to water.

Rats were kept in individual wire screen metabolism cages. Urine specimens were collected at 24 hour intervals and analyzed daily. Each collecting flask contained 1 gm. of citric acid. Feces were analyzed in some experiments; they were collected daily, kept in 30% sulfuric acid, and the N-content was determined on the pooled specimens of each control and experimental period.

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Nitrogen was determined in urine, feces, and diet by a micro-Kjeldahl procedure.

Rats were kept in a constant temperature room (approximately 72°F), and each was studied for a control period of 7 days, followed by an experimental period of equal length. All manipulations (weighing, injections) were performed daily at the same time.

Upon completion of the experiment all animals were killed and a complete autopsy performed. In the case of the thyroidectomized rats a careful search was made for thyroid remnants. The completeness of hypophysectomy was judged by inspection of the fossa and by gross and microscopic study of thyroids, testes, and adrenals. Any tissue suspect of being a thyroid or pituitary remnant was sectioned and examined microscopically. Experiments on animals incompletely thyroidectomized or hypophysectomized were discarded.

TABLE 1. EFFECT OF THYROXINE ON NITROGEN EXCRETION OF THYROIDECTOMIZED RATS

Exp. No.	Daily dose of thyroxine μg.	Sex	Mean urinary N excretion mg. per 24 hrs.*		Diff. mg.	"P" of Diff.
			Control period	Treatment period		
1	10	M	223	188	−35	0.01
2	5	M	224	180	−44	0.01
3	5	M	256	199	−57	0.01
4	5	M	214	162	−52	0.01
5	5	M	202	169	−33	0.01
6	5	M	256	202	−54	0.01
7	5	M	176	131	−45	0.01
8	5	M	183	155	−28	0.01
9	5	M	173	130	−43	0.01
10	5	M	168	148	−20	0.02
11	5	M	209	161	−48	0.01
12	5	M	168	156	−12	0.4
13	5	F	158	137	−21	0.01
14	5	F	151	129	−22	0.01
15	5	F	167	143	−24	0.01
16	50	M	232	252	+20	0.01
17	50	M	153	179	+26	0.01
18	50	M	155	179	+24	0.01

* Includes the first 24 hrs. of the latent period.

RESULTS

In 14 of 15 thyroidectomized rats injected daily for 7 days with either 5 or 10 micrograms of thyroxine the urinary output of N decreased significantly. There was no significant change in weight during this period. When 50 or 100 micrograms of thyroxine were injected daily, N-excretion increased. Both the anabolic effect of the small doses and the catabolic effect of the larger doses became evident on the third day of treatment (Table 1).

When rats were fed a constant intake prior to and immediately following thyroidectomy, the "protein catabolic" effect of thyroidectomy became evident. The N-excretion, which was 78% of the N intake prior to operation rose to 88% after thyroidectomy and fell to 62% following replacement therapy with 5 micrograms of thyroxine (Table 2).

TABLE 2. INFLUENCE OF THYROIDECTOMY AND OF SUBSEQUENT THYROXINE TREATMENT ON N BALANCE

Rat No.	N-intake mg. per 24 hrs.	Control period			Post-operative period[5]			Treated[6]		
		Urinary N excretion mg. per 24 hrs.	% intake excreted	Weight change gm.	Urinary N excretion mg. per 24 hrs.	% intake excreted	Weight change gm.	Urinary N excretion mg. per 24 hrs.	% intake excreted	Weight change gm.
1[1]	280	216 ± 8.7[4]	77	+10	215 ± 53	77	+6	205 ± 7	74	+10
2[1]	280	237 ± 20	84	+ 2	239 ± 10	85	+1	232 ± 11	83	+ 3
3[2]	280	235 ± 10.9	84	+ 5	262 ± 9.6	94	−5	181 ± 10.3	65	+16
4[2]	280	209 ± 16.3	75	+ 7	228 ± 26	80	+1	172 ± 10.6	61	+10
5[2]	280	213 ± 19	77	+ 5	254 ± 15	90	−5	172 ± 6.5	61	+10
6[2]	280	213 ± 19	77	+ 5						
7[2]	280	214 ± 6.2	77	+ 6						
8[2]	280	209 ± 15.3	75	+ 8						
M			78			88[7]				

[1] Sham operated.
[2] Thyroidectomized.
[3] Died at operation.
[4] Standard deviation.
[5] 14th–21st day post-operation.
[6] Thyroxine (5 μg.) daily.
[7] (P of difference less than 0.02.)

In contradistinction to the protein anabolic (N sparing) effect of small doses of thyroxine in the thyroidectomized animal, thyroxine in amounts of 5–10 micrograms had no influence upon N-balance in the intact rat. 50 or 100 micrograms of thyroxine, however, caused an increased output of urinary nitrogen, and weight loss.

Hypophysectomized rats injected with 5 micrograms of thyroxine for 7 days showed no change in N-excretion or weight. 100 micrograms of thyroxine injected daily for 7 days failed to induce an increase of N excretion. In three of the nine rats injection of the large dose of thyroxine was followed by a slight decrease in N excretion (Table 3).

Fecal nitrogen and fluid intake and output were not changed by any of the experimental procedures used.

TABLE 3. EFFECT OF THYROXINE IN HYPOPHYSECTOMIZED RATS

Exp. No.	Daily dose of thyroxine γ	Mean urinary N excretion mg. per 24 hrs.*		Diff. mg.	"P" of Diff.
		Control period	Treatment period		
1	None	198	193	− 5	0.6
2	None	199	203	+ 4	0.7
3	None	205	206	+ 1	0.9
4	None	216	208	− 8	0.3
5	None	192	194	+ 2	0.8
6	5	197	204	+ 7	0.4
7	5	192	199	+ 7	0.3
8	5	185	197	+12	0.1
9	5	230	220	−10	0.3
10	5	207	216	+ 9	0.2
11	5	195	192	− 3	0.7
12	100	175	181	+ 6	0.3
13	100	197	181	−16	0.02
14	100	193	180	−13	0.02
15	100	190	179	−11	0.02
16	100	186	179	− 7	0.2
17	100	192	186	− 6	0.3
18	100	191	189	− 2	0.7
19	100	186	186	0	1
20	100	184	190	+ 6	0.4

* Includes first 24 hours of the latent period.

DISCUSSION

That thyroid hormone exerts a protein anabolic effect under certain circumstances was suggested by observation that young thyroidectomized rats (Evans, Simpson, and Pencharz 1939) and goats (Reineke and Turner 1941) and human cretins resume growth under treatment with thyroid hormone. A greater growth rate of intact mice given thyroxine has been demonstrated; in the latter experiments, however, the weight increase was almost completely accounted for by increased food intake (Koger, Hurst, and Turner 1942). Our experiments were carried out on force-fed rats, thus obviating the difficulties of interpretation resulting from change in food intake following thyroid administration in ad lib. fed animals. Studies of N-balance in human hypothyroids with food intake as nearly controlled as possible have suggested increased N-retention under thyroid medication (Janey 1918, Johnston and Maroney 1939, Wilkins 1945) but in long-term clinical experiments the maintenance of a constant intake is difficult to enforce.

Our experiments demonstrate that the thyroidectomized rat under conditions of constant intake (force-feeding) retains less N than does the normal rat, either because it cannot anabolize protein or because simultaneously occurring catabolic processes are increased. The finding of increased N excretion following thyroidectomy is in agreement with the results of Persike (1948). It should be stressed that the studies on thyroidectomized rats were performed three weeks after operation except as noted in Table 2. Consequently, the increased N excretion cannot be interpreted as resulting from an unspecific alarm reaction. Furthermore, N excretion following other relatively simple operations in the rat is either only slightly increased for a short time or shows no significant change.

Daily injection of 5–10 micrograms of thyroxine decreases the nitrogen excretion of the thyroidectomized rat, apparently even to a level lower than that of the intact animal. The secretion of thyroid hormone by the rats thyroid has been calculated to be approximately 5–10 microgram equivalents of thyroxine per day (Dempsey and Astwood 1943, Reineke, Mixner and Turner 1945). The fact that administration of such amounts of thyroxine exert a protein-anabolic effect suggests that the latter may be the physiological action of thyroid hormone.

On the other hand, large amounts of thyroxine, 50 or 100 micrograms per day, induce protein catabolism in intact and in thyroidectomized rats under the conditions of our experiments. In ad lib. fed rats, certain anabolic processes in the liver have been observed after a single injection of large amounts of thyroxine, 400 micrograms per 100 gm. body-weight (Sternheimer 1939.) The experimental conditions in Sternheimer's experiments and in ours differ too widely to permit close comparison but the possibility of anabolic processes in

the liver occurring simultaneously with an overall negative nitrogen balance is of some interest.

The protein anabolic effect of physiological doses of thyroxine was absent in the hypophysectomized rats. Since the hypophysectomized rat is physiologically almost "athyroid," one would expect an anabolic effect such as was observed in the thyroidectomized rats. This suggests that pituitary function may be a prerequisite for the protein anabolic effect of thyroid. This would be in agreement with the observation that the thyroidectomized-hypophysectomized rat does not resume growth under treatment with thyroid hormone (Evans, Simpson and Pencharz 1939). The nature of this interaction or-synergism between thyroid hormone and the pituitary cannot be interpreted at this time. It is probably significant in this connection that in our experiments (on fed hypophysectomized animals) large doses of thyroxine failed to induce protein catabolism whereas protein catabolism has been induced by thyroxine in the fasting hypophysectomized dog (Soskin, Mirsky, Zimmerman and Crohn 1938) and in the fasting hypophysectomized phlorhizinized rat (Wells and Chapman 1941).

SUMMARY

Experiments are reported on the influence of thyroxine on N excretion in force-fed rats kept on a constant food intake.

Thyroidectomy in the rat is followed by an increased output of urinary N. This is a late effect not to be interpreted as unspecific post-operative negative N balance (alarm reaction).

Small, physiological doses of thyroxine decrease N excretion in the thyroidectomized rat (protein anabolic effect). In the intact rat such doses of thyroxine have no influence on N balance.

Large amounts of thyroxine increase N excretion both in the intact and in the thyroidectomized rat (protein catabolic effect).

In the hypophysectomized rat small doses of thyroxine fail to exert a protein anabolic effect and large doses fail to exert a protein catabolic effect.

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LACK OF EFFECT OF LACTOGENIC HORMONE UPON ORGAN WEIGHTS, NITROGEN AND PHOSPHORUS BALANCE, AND THE FAT AND PROTEIN CONTENT OF LIVER AND CARCASS IN MALE RATS GIVEN LACTOGENIC HORMONE

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PRIOR TO THE isolation of the lactogenic hormone of the anterior pituitary in homogeneous form there were many reports that nonpurified preparations of this hormone exerted metabolic effects not directly associated with its role in lactation and luteotrophic action. The biologic properties of pure lactogenic hormone have not been fully studied. We have been especially interested in the report of Reiss (1947) that a preparation of lactogenic hormone caused a significant decrease in the fat of carcass and skin in the rat. In the present study the administration of lactogenic hormone for 10 days to the force-fed male rat failed to cause any significant change in the weights of liver, stomach, intestines, kidneys, testes, thymus and adrenals, in nitrogen and phosphorus balance, or in the fat and protein content of the liver and carcass.

METHODS

Male rats of the Sprague-Dawley strain were maintained on a diet of Archer Dog Pellets until they reached a weight of approximately 300 gm. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.) The technique of force-feeding and the diets used were modifications of those described by Reinecke, Ball and Saumels (1939). The diet was made according to Table 1. During the period of adaptation to force-feeding the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc. per day on the 5th day.

The animals were housed in an air-contidioned room in which the temperature was maintained at 74 to 78 degrees F and the humidity at 30 to 35 per cent of saturation. Twenty-four-hour samples of urine were collected at the same hour each day and were preserved with thymol and 1 gram of

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TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	Grams
Cellu flour (Chicago Dietetic Supply)	120
Osborne & Mendel salt mixture	40
Diet yeast (Pabst)	100
Wheat germ oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
Water to make total of	2000 cc.

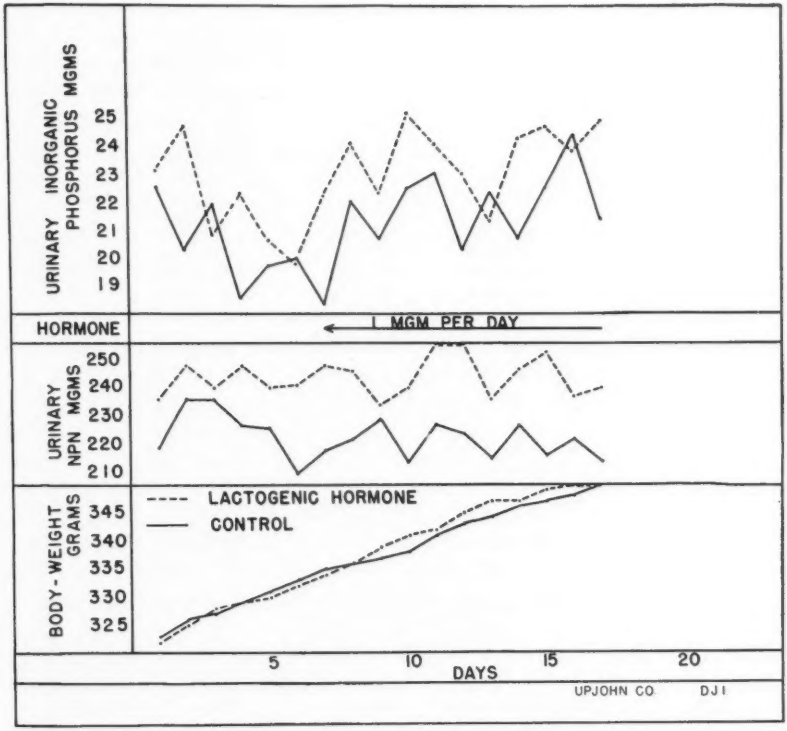

FIG. 1. Lack of effect of lactogenic hormone upon body-weight and urinary nitrogen and phosphorus in normal force-fed male rats. Averages.

citric acid to insure the acidity of the urines for nitrogen analysis. The following methods of analysis were used: Urinary inorganic phosphorus, Müller (1935); urinary non-protein nitrogen by the micro-Kjeldahl procedure; tissue fat and tissue protein, Li *et al.* (1948).

The lactogenic hormone was prepared by the procedure of Li, *et al.* (1942). Following control periods of 14 days, 8 experimental animals were each given 1 mgm. of lactogenic hormone by subcutaneous injection in 3 divided doses per day for 10 days. Eight control animals were given injections of physiological saline. At the end of the experiment the animals were anesthetized with ether and exsanguinated. The hair was carefully shaved from the body, all of the intra-thoracic and intra-abdominal organs were removed, and the carcass including skin was chilled but not frozen, ground and lyophilized at low temperature until dry. The liver was not ground but was subjected to lyophilization. The carcass and liver were then analyzed for fat and protein.

RESULTS

The data on body-weight, urinary non-protein nitrogen and inorganic phosphorus are in Figure 1, the data on organ weights are in

TABLE 2. ORGAN WEIGHTS FROM LACTOGENIC HORMONE EXPERIMENT

Organ	Lactogenic Hormone		Controls	
	Average	Range	Average	Range
Liver	9.95 gms.	8.95–10.85	9.96 gms.	8.40–11.00
Stomach	1.52 gms.	1.45– 1.60	1.53 gms.	1.40– 1.83
Small Intestine	5.95 gms.	5.20– 6.45	5.89 gms.	5.40– 6.34
Cecum and Colon	2.11 gms.	1.85– 2.30	2.13 gms.	2.00– 2.30
Kidneys	2.29 gms.	2.10– 2.40	2.17 gms.	1.85– 2.50
Testes	3.29 gms.	2.98– 3.55	3.09 gms.	2.70– 3.58
Thymus	242 mgms.	105–334	269 mgms.	184–330
Adrenals	44 mgms.	38–48	46 mgms.	40–52

TABLE 3. LIVER COMPOSITION OF RATS TREATED WITH LACTOGENIC HORMONE. AVERAGES AND RANGE

		Composition in Gms. per 100 Gms. Wet Liver		
	Liver Wt. Gms.	Water	Fat	Protein
Lactogenic hormone	9.95 (8.95–10.85)	67.68 (66.9–68.6)	5.39 (4.60–6.86)	21.08 (19.0–22.2)
Control	9.96 (8.40–11.00)	67.55 (66.9–68.5)	4.46 (2.86–5.92)	22.06 (20.8–23.3)

TABLE 4. CARCASS COMPOSITION OF RATS TREATED WITH LACTOGENIC HORMONE AVERAGES AND RANGE

		Composition in Gms. per 100 Gms. Wet Carcass		
	Body Wt. Gms.	Water	Fat	Protein
Lactogenic hormone	352 (339–363)	68.67 (67.3–69.5)	11.55 (8.6–13.6)	15.36 (14.1–16.9)
Control	350 (334–367)	68.33 (65.9–70.7)	12.19 (9.3–14.6)	15.23 (14.1–16.2)

Table 2, the data on liver composition are in Table 3 and the data on carcass composition are in Table 4. The administration of lactogenic hormone did not cause any significant change in any of the indices applied in this study. The animals treated with lactogenic hormone excreted more nitrogen than did their controls but his group difference was present during the pre-injection period so that it cannot be said that lactogenic hormone modified nitrogen balance.

DISCUSSION

The differences between the negative results of this study and the positive effects of lactogenic hormone reported by Reiss (1947) must be due either to the presence of biologically active substances other than lactogenic hormone in the extracts used by Reiss or to differences in experimental procedures or the responsiveness of the test animals. Riddle and associates (1947) have summarized their extensive investigations of the biologic effects of "prolactin" in mammals and birds. They found that "prolactin" increases the weight of the liver and pancreas in doves and pigeons and increases the percentage of hepatic glycogen and fat in the pigeon. These actions were found to be absent or negligible in the rat and other mammals.

The data of Figure 1 shows a consistent average difference in the amounts of nitrogen excreted by the two groups during both the pre-injection and the experimental periods. This illustrates the advisability of using the data obtained during the control period to divide the animals into more precisely balanced groups than is likely to be attained by random selection.

SUMMARY

Male rats of approximately 300 grams initial weight were force-fed a medium carbohydrate fluid diet. Following a control period of 14 days, 8 rats were given 1 mgm. of pure lactogenic hormone in 3 divided injections per day for 10 days. An equal number of control rats were given injections of physiological saline. Nitrogen and phosphorus were determined in urine during the experiment. At necropsy the liver, stomach, intestines, kidneys, testes, thymus and adrenals were weighed and the fat and protein content of the liver and carcass were determined. The results were negative.

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STUDIES ON THE NATURE OF THE PROTEIN CATABOLIC RESPONSE TO ADRENAL CORTICAL EXTRACT[1,2]

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IT IS GENERALLY believed that in addition to their other actions, those adrenal cortical steroids which influence protein metabolism do so by increasing the rate of protein catabolism. The coupling of the increase in nitrogen excretion with an accumulation of liver glycogen following treatment with adrenal cortical hormones in the fasted animal has led to the concept that these steroids are concerned with the process of gluconeogenesis from protein (Long, Katzin, and Fry, 1940). These findings, amply substantiated by many different investigators, have directed attention toward various steps in the metabolic cycle between protein and glycogen as possible sites for the locus of action of the adrenal cortex in metabolism. The degradation of protein itself, the deamination of amino acids, the conversion of three carbon precursors to glucose and glycogen, the conversion of glucose to glycogen, and the inhibition of extra hepatic glucose utilization have all been considered in terms of the site of action of the cortical steroids in metabolism (Long 1942). The purpose of this study is to attempt to throw further light on the mechanism of intervention of the adrenal cortex in protein metabolism. In a previous study (Engel, Pentz, and Engel, 1948) a method was developed for measuring quantitatively small changes in protein metabolism over brief periods. The method depends on the relatively steady rate of urea synthesis that occurs during the first 18 to 24 hours following bilateral nephrectomy in the rat. Since urea is equally distributed throughout the body water, changes in the blood urea nitrogen of the nephrectomized rat after various experimental procedures quantitatively reflect increases or

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[2] Preliminary reports of portions of this work have appeared in abstract form, Engel, Pentz and Engel 1947 and Engel, Schiller, Pentz and Bondy 1948.

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decreases in protein catabolism, urea being the end product of this process.

By application of this technique to the study of cortical hormone action it has been possible to determine the time following injection at which the protein catabolic action of adrenal cortical extract becomes apparent and to measure quantitatively its magnitude. The effect of various experimental procedures on this action of cortical hormone will be considered.

MATERIALS AND METHODS

Part of these experiments were carried out at Emory University and part at Duke University.

In the Emory series male rats of the Sprague-Dawley strain, weighing 180 to 240 grams were used. In the Duke series Vanderbilt strain male rats from 160 to 220 grams in weight were used. The former were fed Rockland rat chow, the latter Purina dog chow supplemented with liver pudding (pork liver 25, cornmeal 25, cracked wheat 25, powdered milk 3 and NaCl 1.4) and lettuce each twice a week. The Emory group were kept in an air-conditioned room from 24–27° C., the Duke group were not. The technique for studying nitrogen metabolism in the nephrectomized rat and the analytical methods have been described in previous reports (Engel and Engel, 1947, Engel, Pentz, and Engel, 1948). All results are expressed in milligrams of urea nitrogen synthesized per 100 grams body weight per hour, comparing a three hour control period with an equivalent experimental period. The animals were fasted for 16 hours following nephrectomy and prior to the experimental period. With the exception of the adrenal cortical extract which was given subcutaneously, all test substances were given intravenously via the external saphenous vein. The animals were kept under light nembutal anesthesia during the experimental period.

TABLE 1. COMPOSITION OF AMINO ACID MIXTURES

	VuJ-n mg. per cc.	mg. N per cc.	"Non-Glycogenic VuJ" mg. per cc.	mg. N per cc.
Glycine	8.0	1.49	—	—
dl-threonine	8.6	0.95	l-threonine 2.0	0.2
dl-valine	11.1	1.33	l-valine 6.5	0.87
l-histidine-HCL	3.2	0.64	l-histidine-HCL-H_2O 3.4	0.68
l-arginine-HCl	6.4	1.70	l-arginine-HCl 2.5	0.81
dl-phenylalanine	5.5	0.47	l-phenylalanine 6.0	0.50
dl-methionine	4.9	0.45	l-methionine 3.8	0.35
dl-isoleucine	8.6	0.91	l-isoleucine 7.0	0.75
dl-leucine	12.3	1.31	l-leucine 15.0	1.60
l-lysine-HCl	9.9	1.34	l-lysine-HCl-H_2O 61.2	8.53
dl-tryptophane	1.5	0.20	l-tryptophane 2.0	0.27
Total	80.0	10.79	109.4	14.56
At a dose of 0.87 ml. per 100 gms. (9.4 mg. N) 70% (5.5 mg.) of the utilizable N (7.8 mg. N) comes from glycogenic amino acids and 30% (2.3 mg.) from non-glycogenic amino acids.			At a dose of 0.81 ml. per 100 gms. (11.8 mg. N) 23.6% (2.8 mg.) of the N comes from glycogenic amino acids and 76.4% (9.0 mg.) from non-glycogenic amino acids.	

Liver glycogen levels were measured by a modification of the method of Good, Kramer and Somogyi (1933), with glucose determined by the photometric technique of Nelson (1944).

All test materials were injected as sterile solutions. The following substances were used:

1. VuJ-n (Merck and Company). An 8 per cent solution of synthetic amino acids consisting of the 10 essential amino acids plus glycine in the proportions listed in Table 1. This was used at a dose of 0.87 ml. or 9.4 mg. N per 100 gms. body weight. 70 per cent of the nitrogen in this mixture came from glycogenic amino acids. With the exception of glycine, histidine and arginine all the amino acids in this mixture were in the racemic form. On the basis of present information as to the utilization of d-amino acids in the rat, it was estimated that 83 per cent of the injected N could be used (Albanese, 1947).

2. "Non-glycogenic VuJ." A mixture prepared by ourselves from material supplied by Merck and Company and having the composition recorded in Table 1. But for glycine, it contained the same amino acids as the VuJ-n but all were of the natural form. The six mono-amino mono-carboxylic acids (l-isoleucine, l-leucine, l-methionine, l-phenylalanine, l-threonine and l-valine) were in form of a specially prepared casein hydrolysate and contained a small proportion of unidentified nitrogen. The solution was made up to a concentration of 10.9 per cent, adjusted to pH 7.05, autoclaved and injected at a dose of 0.81 ml. or 11.8 mg. N per 100 grams body weight. 23.6 per cent of the nitrogen in this mixture was derived from glycogenic amino acids. It should be noted that lysine was the chief non-glycogenic amino acid in the mixture.

3. Lysine-hydrochloride 6.85 per cent adjusted to pH 7.4 and autoclaved. Dose 1 ml. per 100 grams body weight (50 mg. free lysine or 9.6 mg. N).

4. 5 per cent glucose in normal saline. Dose 1 ml. or 50 mg. glucose per 100 gms. body weight.

5. VuJ-n in 5 per cent glucose in distilled water. Dose 0.87 ml. per 100 gms. body weight.

6. Human serum albumin, salt free, diluted to 6.25 per cent with normal saline. Dose 1 ml. or 10 mg. N per 100 gms. body weight.

7. 6.25 per cent human serum albumin in 5 per cent glucose in saline. Dose 1 ml. per 100 gms. body weight.

8. 15 per cent corn oil emulsion stabilized with 3 per cent soy bean phosphatides prepared by Dr. F. J. Stare. Diluted to 3 per cent with normal saline and administered at a dose of 1 ml. or 30 mg. of fat per 100 gms. body weight.

9. Upjohn's aqueous adrenal cortex extract.

RESULTS

The results to be recorded represent data collected in two laboratories from different strains of rats kept under different dietary and environmental conditions. Although the basal hourly rates of urea formation calculated for a three hour period beginning 15–17 hours after nephrectomy were different in the two strains, the responses to the experimental procedures were qualitatively and quantitatively

similar in all cases compared. In 201 rats of the Emory series the basal rate was 3.0 ± 0.08 mg. N per 100 gms. per hour (Engel, Pentz and Engel, 1948) while in 243 rats of the Duke series the rate was 2.0 ± 0.05 mg. N per 100 gms. per hour. A third series of animals reported in the accompanying paper and studied in the Emory laboratory were of the Rockland strain and fed a low potassium diet. These animals had a basal rate of urea formation of about 1.8 mg. N per 100 gms. per hour (Bondy, Engel, and Farrar, 1949). The laboratory of origin of each experiment is recorded in each table.

Effects of Adrenal Cortical Extract

Adrenal cortical extract (ACE) was injected subcutaneously in two equal doses at the beginning and end of the first hour of the control period. Blood was collected at 0, 3, and 6 hours (16, 19, and 22 hours after nephrectomy) and the average hourly rates of urea for-

TABLE 2. INFLUENCE OF A.C.E. ON UREA N PRODUCTION IN THE NEPHRECTOMIZED RAT

Procedure*	No. of rats	Rate of urea nitrogen formation in mg. N per 100 gm. body wt. per hr.			
		0–3 hrs.	3–6 hrs.	Change	"p"
Saline (1.0 ml. per 100 gm.)	10	1.7 ± 0.15	1.9 ± 0.17	$+0.2 \pm 0.20$	>0.5
A.C.E. (0.5 ml. per 100 gm.)	11	1.9 ± 0.20	2.6 ± 0.23	$+0.7 \pm 0.25$	<0.02
A.C.E. (0.8 ml. per 100 gm.)	17	1.8 ± 0.22	2.6 ± 0.28	$+0.8 \pm 0.29$	<0.01
A.C.E. (1.0 ml. per 100 gm.)	22	2.2 ± 0.20	3.0 ± 0.27	$+0.8 \pm 0.24$	<0.01
A.C.E. (2.0 ml. per 100 gm.)	12	1.8 ± 0.20	2.8 ± 0.27	$+1.0 \pm 0.32$	<0.01

* Duke Series—Basal Rate 2.0 ± 0.05 mg. N per 100 gms. per hour.
The values in this and subsequent tables represent the means with their standard errors.

mation calculated for the 0–3 and the 3–6 hour periods. A control series in which 1 ml. of normal saline was injected is included. Table 2 records the results. Note that the saline was without effect on the rate of urea formation. Regardless of dose, the rate of urea formation during the first three hours following the injection of ACE was not significantly different from the basal control rate of 2.0 ± 0.05 mg. N per 100 gms. per hour. This confirms previously reported data using a different strain of rats (Engel, Pentz and Engel, 1948).

During the second three hour period a highly significant increase in urea formation occurred with all doses of ACE used except the smallest. Within the dose range employed there was no clear-cut correlation between the dose and response once a significant increment

was achieved. From these studies it is apparent that the protein catabolic effect of ACE in the fasted nephrectomized rat begins about 3 hours after injection and amounts to about 1 mg. of N per 100 gms. body weight per hour for the subsequent 3 hours under the conditions of these experiments. This may be compared to the increase of 2.2 mg. of total urinary nitrogen per 100 gms. body weight per hour found by Long, Katzin, and Fry (1940) following 12 consecutive hourly injections of 1 ml. of ACE into normal fasted rats and 0.7 mg. of urinary nitrogen per 100 gms. per hour for 24 hours after 8 hourly injections of 1 ml. each of adrenal cortical extract found by Noble and Toby (1948). No attempts have been made to determine how long the effect lasts in our experiments. A similar stimulation of protein catabolism occurs after ACE in the adrenalectomized-nephrectomized rat (Bondy and Engel, 1947).

Effects of Amino Acids on Protein Catabolic Action of ACE

If the adrenal cortex were concerned primarily with the conversion of amino acids to glycogen, or if it had an obligatory effect on endogenous protein catabolism, one might anticipate that the administration of amino acids at a time when ACE was exhibiting its effect on nitrogen metabolism would result in an enhanced rate of urea formation. The data in Table 3 shows that this is not the case. 9.4 mg. of N in the form of a mixture of amino acids (Merck's VuJ-n) injected intravenously at the end of the third hour increased the rate of urea N formation 1.2 ± 0.18 mg. per hour during the subsequent 3 hours. When combined with a small dose of ACE (0.4 ml. per 100 gms.) which itself caused no significant stimulation of protein catabolism, 1.3 ± 0.20 mg. of urea N per hour appeared during the following 3 hours. Administration of VuJ-n to rats which had received doses of ACE causing increases in urea N of the order of 1.2 ± 0.28 and $0.9 \pm$

TABLE 3. INFLUENCE OF INTRAVENOUS AMINO ACIDS ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

Procedure*	No. of Rats	Urea N mg. N per 100 gm. body wt. per hour			"p"
		0–3 hrs.	3–6 hrs.	Change	
VuJ-n (9.4 mg. N per 100 gm.)	24	3.1±0.23	4.3±0.23	+1.2±0.18	<0.01
A.C.E. (0.4 ml. per 100 gm.)	18	3.0±0.21	3.4±0.26	+0.4±0.23	0.2
A.C.E. (0.4 ml. per 100 gm. plus VuJ-n 9.5 mg. N/100 gm.)	10	3.0±0.31	4.3±0.15	+1.3±0.20	<0.01
A.C.E. (0.8 ml. per 100 gm.)	13	2.9±0.22	4.1±0.34	+1.2±0.28	<0.01
A.C.E. (0.8 ml. per 100 gm. VuJ-n 9.4 mg. N per 100 gm.)	12	2.6±0.35	3.9±0.27	+1.3±0.32	<0.01
A.C.E. (1.2 ml. per 100 gm.)	12	2.8±0.21	3.7±0.26	+0.9±0.23	<0.01
A.C.E. (1.2 ml. per 100 gm. VuJ-n 9.4 mg. N per 100 gm.)	10	2.8±0.26	4.0±0.19	+1.2±0.22	<0.01
VuJ-n (9.4 mg. N per 100 gm. i.v., 9.4 mg. N per 100 gm. i.p)	9	3.4±0.20	6.1±0.31	+2.7±0.26	<0.01

* Emory Series—Basal Rate 3.0±0.08 mg. N per 100 gm. per hour.

0.23 mg. N per hour, yielded no greater amount of urea than if either ACE or the VuJ-n alone were injected. The increases were 1.3 ± 0.32 and 1.2 ± 0.22 mg. N per 100 gms. per hour, respectively. This represents conversion to urea of approximately 46 per cent of either the injected nitrogen or its equivalent from the tissues. This is not the limit of the liver's ability to deaminate amino acids under these circumstances as doubling the dose of amino acids approximately doubled the subsequent yield of urea. This is recorded in the last line of Table 3. The administration of 18.8 mg. of N per 100 gms. resulted in the formation of 2.7 ± 0.27 mg. of extra urea N per hour for the subsequent 3 hours.

The failure to get an additive effect on urea formation when ACE and amino acids were administered concurrently argues against deamination of amino acids with subsequent urea formation as being a necessary result of cortical hormone action. By the same token, however, these results can be taken as evidence against a primary action on protein breakdown by ACE. One would anticipate that the amino acids resulting from this reaction would be added to the amino acid pool available for deamination by the liver, unless the high concentration of amino acids resulting from the injection suppresses endogenous protein catabolism. The following experiment suggests that the latter may be the case. In a previous study (Engel and Engel, 1946) it was shown that following the intravenous injection of amino acids into the nephrectomized rat most of the resulting urea was formed in the first hour, less in the second and very little in the third. Although the total amounts of urea formed in 3 hours after ACE, VuJ-n and ACE plus VuJ-n were the same it seemed possible that a difference in the rates of urea formation after each of these substances might be detected if measured each hour under these circumstances. The results are shown in Table 4.

The average hourly rates of urea formation for the control and experimental periods are recorded in the first part of the table. The second part indicates the excess urea above the basal rate formed during the 4th, 5th, and 6th hours respectively. Due to the greater amount of sampling and deeper anesthesia with resultant greater tendency to respiratory obstruction from mucus, a small but just significant increase in urea formation occurred during the second three hour period in the control animals. This did not occur in other experiments in which samples of blood were taken only at 3 hour intervals. The increase was of probable significance during the 3rd to 4th hour ($p < 0.05$) but not during the last two hours. The stimulation of urea formation by mild hemorrhage and anoxia has been noted before (Engel & Engel, 1946). It is reflected in subsequent experiments with ACE and VuJ-n by a somewhat greater urea formation than recorded in the previous experiment in which less bleeding was done. In the ACE treated rats a significant increase in urea formation occurred,

TABLE 4. UREA SYNTHESIS EACH HOUR AFTER A.C.E. AND AMINO ACID TREATMENT IN NEPHRECTOMIZED RATS

Procedure*	No. of rats	Mg. Urea N per 100 gm. body wt. per hour			Urea N formed in excess over control period Mg. N per 100 gms.					Liver glycogen Mg. per 100 gms. liver
		Control		Exp.						
		0–3 hrs.	"p"†	3–6 hrs.	(4th hr.)	"p"†	(5th hr.)	"p"†	(6th hr.)	
Control	14	1.8 ±0.23	<0.05	2.7 ±0.27	1.2 ±0.49 p <0.05‡	0.5	0.7 ±0.48 p =0.2‡	0.5	0.3 ±0.30 p =0.5‡	283 ±34
A.C.E. (0.8 ml. per 100 gm.)	16	1.8 ±0.22	<0.01	3.4 ±0.31	2.2 ±0.54 p <0.01	0.4	1.6 ±0.50 p <0.01	0.5	1.3 ±0.38 p <0.01	231 ±37
VuJ-n (10.4 mg. N per 100 gm.)	17	1.9 ±0.18	<0.01	4.1 ±0.25	3.9 ±0.62 p <0.01	<0.05	2.2 ±0.52 p <0.01	<0.02	0.5 ±0.41 p =0.3	483 ±88
A.C.E. (0.8 ml. per 100 gm.) VuJ-n (10.4 mg. N per 100 gm.)	15	2.4 ±0.20	<0.01	3.9 ±0.19	3.2 ±0.48 p <0.01	<0.01	1.0 ±0.32 p <0.01	0.5	0.5 ±0.43 p =0.2	546 ±77

* Duke Series—Basal Rate 2.0 ±0.05 mg. N per 100 gms. per hour.
† The "p" values refer to the probability of significance between each column.
‡ The "p" values below refer to the probability of significance compared to the 0–3 hour basal rate.

as before. The excess amounts of urea formed each hour represented a significant increase over the basal rate, but are not significantly different from each other. If these rates are corrected for the non-specific stimulation by subtracting the excess urea appearing each hour in the control series they become 1.0 mg., 0.9 mg., and 1.0 mg. N per 100 gm. during the 4th, 5th, and 6th hours respectively. Following amino acid injection significant increases in urea formation occurred during the 4th and 5th hours, but not the 6th and the rates each hour were each significantly less than the previous one. Corrected for the non-specific stimulation these rates become 2.7, 1.5, and 0.2 mg. N per 100 gms. When ACE and VuJ-n were given the results were identical with those following VuJ-n alone and in sharp contrast with the relatively steady rate of urea formation after ACE alone. The corrected values for the increases in urea formation were 2.0, 0.3, and 0.2 mg. N per 100 gms. per hour. While the responses to amino acids and to ACE plus amino acids were the same in that the excess in urea formation each hour was significantly less than the preceding hour, none of these were significantly different from the corresponding hour in the ACE series, in which the excess urea formed each hour was approximately the same. In view of the larger error inherent in taking hourly samples this is not surprising in series of this size. Limited significance can be attached, therefore, to the experiment as a whole. Considering each response by itself it can only be suggested that the response to ACE and amino acids is more as if amino acids alone were given, i.e. that the amino acids suppressed the action of the ACE. A significant increase in liver glycogen occurred in the rats treated with amino acids and ACE, but not with ACE alone. Since the apparent suppression of the protein catabolic effect of ACE occurred under circumstances in which there was an increase in liver glycogen the possibility that the availability of gluconeogenetic material may determine whether endogenous protein breakdown takes place after ACE treatment was considered. The experiments recorded in Table 5 were therefore carried out.

The greatest proportion of nitrogen (70 per cent) in VuJ-n comes from glycogenic amino acids. Using the same amino acids except for glycine, a mixture was prepared in which the proportions were so changed that 76.4 per cent of the N now came from non-glycogenic amino acids (Table 1). This mixture, when given in a dose of 11.8 mg. N per 100 gms. body weight was less well utilized or tolerated than the VuJ, the equivalent of 61 per cent of the injected nitrogen appearing as urea in 3 hours. When injected into the ACE-treated animals, the amount of urea formed was neither significantly greater than that after the amino acids alone, nor significantly less than the expected sum if the effects of the amino acids and ACE were additive (Table 5). There was a small but probably significant ($p < 0.05$) increase in liver glycogen in the rats treated with ACE and "Non-

glycogenic VuJ." Since this mixture still contained some glycogenic amino acids, the experiment was repeated using a single, non-glycogenic amino acid, lysine. This material was probably toxic since when injected at a dose of 9.6 mg. N. per 100 gm., 10.14 extra mg. of urea nitrogen appeared in 3 hours and the liver glycogen was significantly decreased. However, when given with ACE, slightly less urea was formed than with lysine alone, again indicating a suppression of the ACE action on protein catabolism. The liver glycogen after ACE and lysine was significantly greater than after lysine alone, but not greater than the control. It was significantly less than after ACE alone.

TABLE 5. INFLUENCE OF INTRAVENOUS AMINO ACIDS ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

Procedure*	No. of rats	Urea N mg. N per 100 gm. body weight per hr.			"p"	Liver glycogen mg. per 100 gm liver
		0–3 hrs.	3–6 hrs.	Change		
Non-glycogenic VuJ (11.8 mg. N per 100 gm.)	13	2.1±0.29	4.9±0.28	+2.8±0.34	<.01	293± 77
A.C.E. (0.8 ml. per 100 gm.)	17	1.8±0.22	2.6±0.28	+0.8±0.29	<.01	231± 37
A.C.E. (0.8 ml. per 100 gm.) plus non-glycogenic VuJ	13	1.9±0.16	5.1±0.39	+3.2±0.33	<.01	499± 60
Control	9					748±126
A.C.E. (0.8 ml. per 100 gm.)	9					1335±173
Lysine (9.6 mg. N per 100 gm.)	13	2.3±0.20	5.7±0.15	+3.4±0.29	<.01	487± 82
A.C.E. (0.8 ml. per 100 gm.) plus lysine (9.6 mg. N per 100 gm.)	14	2.5±0.27	5.3±0.28	+2.8±0.26	<.01	928± 91

* Duke series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hour.

These results suggest that the inhibitory action of amino acids may not be related directly to their glyconeogenetic potency. However, elevated liver glycogen levels after lysine and ACE raise the possibility that in the presence of ACE, the non-glycogenic amino acids simply exchange with glycogenic amino acids in the tissue. The alleged unreactivity of lysine throws doubt on such a concept (Schoenheimer, 1942). Lysine on the other hand may simply serve as a nonspecific stress in these experiments.

Effects of Glucose on the Protein Catabolic Action of ACE

With the observation that amino acids apparently suppressed the action of ACE on endogenous protein metabolism, it became of interest to see whether other metabolites had a similar action. Table 6 records the effects of glucose. The experiment was performed twice, once in each laboratory, with identical results but only those from the Duke laboratory are recorded. Glucose was administered intravenously in a dose of 50 mg. per 100 grams, a dose approximately isocaloric with that of the amino acid used in the previous experiments. Glucose itself had no effect on urea formation but when given in conjunction with ACE completely abolished the increase in nitro-

gen customarily seen after treatment with this hormone. That the suppression of urea formation is at the level of protein breakdown rather than deamination of amino acids is suggested by the four last experiments in this series in which it is shown that glucose does not prevent the increase in urea that followed the injection of amino acids. This was true both after rapid and slow injection of the amino acids. The rate of delivery of amino acids to the liver therefore is probably not a significant factor in the response to glucose.

A similar effect of glucose on nitrogen metabolism after ACE was noted by Long, Katzin, and Fry (1940) in intact rats given 5 ml. of cortical extract intraperitoneally and 750 mg. of glucose by stomach

TABLE 6. INFLUENCE OF INTRAVENOUS GLUCOSE ON THE UREA PRODUCTION AFTER A.C.E. AND AMINO ACIDS IN THE NEPHRECTOMIZED RAT

Procedure	No. of rats	Urea N Mg. N per 100 gm. body wt. per hr.			"p"
		0–3 hrs.	3–6 hrs.	Change	
#Glucose (50 mg. per 100 gm. i.v.)	6	1.7±0.28	1.8±0.17	+0.1±0.38	0.5
#A.C.E. (0.8 ml. per 100 gm.)	17	1.8±0.22	2.6±0.28	+0.8±0.29	<0.01
#A.C.E. (0.8 ml. per 100 gm.) plus glucose (50 mg. per 100 gm.)	11	2.1±0.18	2.3±0.21	+0.2±0.27	0.5
*VuJ-n (9.4 mg. N per 100 gm.)†	24	3.1±0.23	4.3±0.23	+1.2±0.18	<0.01
*VuJ-n 9.4 mg. N per 100 gm.) +Glucose (50 mg. per 100 gm.)†	14	3.7±0.32	5.4±0.25	+1.7±0.41	<0.01
#VuJ-n (10 mg. N per 100 gm.)‡	8	2.3±0.30	4.2±0.21	+1.9±0.43	<0.01
#VuJ-n (10 mg. N per 100 gm.) plus glucose (50 mg. per 100 gm.)‡	8	1.7±0.31	4.5±0.31	+2.8±0.35	<0.01

* Emory Series—Basal Rate—3.0±0.08 mg. N per 100 gm. per hour.
# Duke Series—Basal Rate—2.0±0.05 mg. N per 100 gm. per hour.
† Test solution injected intravenously over 5 minutes.
‡ Test solution injected intraperitoneally in 6 divided doses over one hour.

tube. No change in nitrogen excretion occurred during the four hours of observation. In a later report (Long, 1942) an increase of only 5.4 mg. of urinary nitrogen per 100 grams in 12 hours was found after 11 hourly injections of 1 ml. of cortical extract were given with three feedings of glucose. This may be compared to the increase of 27 to 33 mg. of N previously reported in fasted normal, adrenalectomized and hypophysectomized rats given a similar dose of cortical extract (Long, Katzin, and Fry 1940).

Effect of Fat on the Protein Catabolic Action of ACE

The availability of a fat emulsion suitable for intravenous administration made it possible to examine the effect of fat on the protein catabolic effect of ACE. This material in the form of a 15 per cent emulsion of corn oil stabilized with 3 per cent soy bean phosphatides was kindly supplied us by Dr. F. J. Stare of the Department of Nutrition, Harvard Medical School. The material was diluted to 3 per cent fat with saline and injected at a dose of 30 mg. of fat per

100 grams body weight. The results are seen in Table 7. Neither the saline control nor the fat itself produced any change in nitrogen metabolism. ACE produced its usual effect and, in contrast to the results with glucose and amino acids, was not inhibited by the fat. There were small rises in liver glycogen in each of the experimental groups compared to the saline controls, but these were not statistically significant. Assuming that the injected fat was metabolized, and we have no proof of this from these experiments, it is apparent that merely supplying calories to the fasting animal is not sufficient to block the ACE effect on protein metabolism.†

TABLE 7. INFLUENCE OF INTRAVENOUS FAT ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

Procedure*	No. of rats	Urea N mg. N per 100 gm. body wt. per hr.			"p"	Liver glycogen mg. per 100 gm. liver
		0–3 hrs.	3–6 hrs.	Change		
Saline (1.0 ml. per 100 gm.)	10	1.7±0.15	1.9±0.17	+0.2±0.20	>0.5	458±133
Fat (1.0 ml. 3.0% in saline)	11	2.3±0.26	2.3±0.20	0.0±0.19	>0.5	709±142
A.C.E. (1.0 ml. per 100 gms.)	10	2.2±0.24	3.0±0.30	+0.8±0.23	<0.01	672± 99
A.C.E. (1.0 ml. per 100 gms.) plus Fat (1.0 ml. 3.0% in saline)	8	1.5±0.31	2.5±0.18	+1.0±0.19	<0.01	683±143

* Duke Series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hr.

The Effects of Protein on the Protein Catabolic Action of ACE

The evidence so far has indicated that ACE does not act primarily in protein metabolism at the level of deamination of amino acids and that such actions as it may have on protein breakdown itself may be masked by the administration of glucose and amino acids. The next consideration was what would be the effect of exogenously administered protein on ACE action. For this purpose salt poor human albumin was used. It was injected at a dose approximately equivalent to that of the amino acids used i.e. 10 mg. N per 100 grams. Since repeated and identical positive results had been achieved with ACE alone the results are compared with an earlier representative series with ACE. In Table 8 it will be seen that the intravenous injection of human serum albumin did not cause any increase in urea formation in the control animals. However, when injected into the ACE treated animals an increase of 1.6 ±0.28 mg. N per 100 grams per hour occurred. This increase was questionably significantly greater than that after ACE alone ($p < 0.05$). This result should be compared to the lack of additive effect seen when amino acids are given to ACE treated rats. No increase in urea formation followed the injection of albumin in 5 per cent glucose into the control rats. Moreover, when ACE was given glucose abolished the extra urea formation previously seen following albumin injection (Table 8).

† Since this manuscript was submitted for publication it was discovered that the fat emulsion as originally made contained 4.5 per cent glucose. In the dilution used, i.e. 3% of the solution contained 9 mg. of glucose per ml., a negligible amount.

TABLE 8. INFLUENCE OF INTRAVENOUS ALBUMIN ON THE UREA PRODUCTION AFTER A.C.E. IN THE NEPHRECTOMIZED RAT

Procedure*	No. of rats	Urer N mg. N 100 gm. body weight per hour			"p"
		0–3 hrs.	3–6 hrs.	Change	
A.C.E. (1.0 ml. per 100 gm.)†	22	2.2±0.20	3.0±0.27	+0.8 ±0.24	<0.01
Human serum albumin (6.25% in saline, 10 mg. N per 100 gm.)	12	2.4±0.21	2.7±0.25	+0.3 ±0.32	>0.5
Human serum albumin (6.25% in saline 10 mg. N per 100 gm.) A.C.E. (1.0 ml. per 100 gm.)	13	1.9±0.13	3.5±0.25	+1.6 ±0.28	<0.01
Human serum albumin (6.25% in 5% glucose and saline, 10 mg. N per 100 gm.	7	1.8±0.20	1.9±0.31	+0.1 ±0.41	>0.5
Human serum albumin (6.25% in 5% glucose and saline, 10 mg. N per 100 gm.)	14	2.0±0.15	2.5±0.28	+0.5 ±0.35	>0.5

* Duke Series—Basal Rate 2.0±0.05 mg. N per 100 gm. per hr.
† See Table 2.

DISCUSSION

The results of these studies confirm the well documented fact that adrenal cortical extract increases the rate of protein catabolism in the fasted animal. An effect was detectable in the experiments as early as 3 hours after its administration. This is consistent with the results from other studies on cortical hormone activity such as dissolution of lymphocytes (Dougherty and White, 1944) but had not been demonstrated previously with respect to nitrogen metabolism. The magnitude of the increase in nitrogen metabolism recorded during the three hours of these experiments was somewhat less than obtained by Long, Katzin, and Fry (1940) who measured the 12 hour urinary nitrogen excretion in rats receiving 1 ml. of cortical extract each hour, but similar to that found by Noble and Toby (1948) using smaller amounts of ACE. In our experiments there was no significant increase in urea formation as the dose of cortical extract was raised, but the possible prolongation of the response beyond 3 hours by larger doses was not investigated.

In these studies the increase in urea formation in the fasted rat after ACE treatment was prevented by an injection of glucose or of amino acids, but not when either fat or whole protein were administered. This could be interpreted as indicating that glucose and amino acid inhibited protein catabolism. However, without the use of a tracer technique it is not possible to distinguish between an actual decrease in protein catabolism and an increase in the rate of metabolism of amino acids by a pathway other than deamination and urea formation. The failure of glucose to alter the rate of urea formation after amino acid injection suggests that deamination is not the locus at which glucose exerts its effect in decreasing urea formation after ACE, but rather that glucose spares protein.

The modification of the protein catabolic response to ACE by the various metabolites injected makes possible further consideration

of both the mode and site of action of the adrenal cortex in protein metabolism. Since the administration of adrenal cortical steroids causes an increase in liver glycogen in both the fasted and the fed animal the possibility may be suggested that the availability of glucose or of glycogen precursors may determine in some way whether an increase in endogenous protein catabolism will occur following ACE treatment. The absence of the increase in protein catabolism after ACE when either glucose or amino acids were given and the failure of fat, a poor glycogen precursor, to influence the catabolic response all would favor such a view. The finding that the non-glycogenic amino acids also apparently masked the protein catabolic action of ACE does not seem to support such an interpretation. However, these latter data must be interpreted with caution. While there was no significant increase in urea formation after ACE plus non-glycogenic VuJ-n, compared to after each alone, neither was the change significantly less than that expected were the two additive. Lysine alone, on the other hand, definitely did not yield an additive effect to that from ACE alone. The choice of lysine for these experiments, however, may not have been the best. It was determined by the availability and high solubility of lysine so that it could be given in a dose comparable to the VuJ-n. According to the data of Schoenheimer (1942) lysine does not exchange its nitrogen like most other amino acids. Furthermore lysine is said not to be deaminated in vitro by liver or kidney slices (Felix and Naka, 1940), although Borsook, Deasy, Hagen-Smit, Keighley and Lowy (1948) have recently reported on the formation of a-amino adipic acid from lysine in liver homogenates. Neuberger and Sauger (1944), furthermore, report that both nitrogen atoms of 1-lysine were converted to urea when fed to rats. In addition, administration of a single amino acid or an unbalanced mixture may throw a "strain" on the normal mechanisms of metabolism, yielding a different result from that when a balanced mixture is given. Both after "non-glycogenic VuJ" and lysine in these experiments a considerably greater amount of urea nitrogen was formed than after the more balanced VuJ-n mixture. This may be interpreted either as indicating a high conversion to urea of the injected amino acids or, what is more likely, a "toxic" effect of the amino acids with resultant stimulation of endogenous protein catabolism. Unless tagged amino acids are used it is impossible to distinguish between these two effects. A stimulation of endogenous protein catabolism by lysine with the release of potentially glycogenic amino acids from the tissues could be a factor in the apparent inhibition of the protein catabolic action of ACE. The increase in liver glycogen after ACE plus "non-glycogenic VuJ" and ACE plus lysine could be taken to support such a view-point. For this reason we cannot interpret these results to mean that amino acids need not be glycogenic to inhibit the protein catabolic action of ACE.

The intravenous administration of fat had no effect on nitrogen metabolism in these experiments and did not effect the response to ACE. Assuming that the fat was utilized during this time, this result would indicate that supplying calories in the form of fat, which is not readily converted to carbohydrate, does not interfere with the ACE action on protein metabolism. A small but not significant increase in liver glycogen occurred after the fat. The studies of McKibbin, Ferry, and Stare (1946) on this fat emulsion clearly indicate that this preparation is utilized by dogs and is capable of maintaining a positive nitrogen balance on a low diet protein. By incorporating radioactive trilaurin into the fat emulsion and measuring the output of radioactive CO_2 in exhaled air Geyer, Chipman and Stare (1948) have demonstrated that this emulsion is utilized immediately following intravenous injection.

Albumin was not converted to urea in 3 hours when injected into the nephrectomized rats, but when given to ACE treated rats an amount of urea was formed which was just significantly greater ($p < 0.05$) than that expected after ACE alone. Without the use of tracers it is of course impossible to determine whether the urea formed arose from the injected albumin or from tissue protein. This result therefore cannot be taken to either support or deny the hypothesis that the availability of glycogen precursors determines whether protein catabolism will take place after ACE treatment. The fact that the extra urea formation after albumin and ACE was abolished when glucose was given with the albumin indicates that this protein catabolism, regardless of origin, is subject to the same influences as is the endogenous protein catabolism which follows ACE treatment.

When taken in conjunction with the finding that the adrenalectomized rat shows the same increment in urea formation after amino acids as does the normal rat and does not convert injected plasma protein to urea (Bondy, Engel, and Farrar, 1949) the above data lead to the conclusion that the adrenal cortex must intervene in protein metabolism at the level of protein itself. This conclusion is supported by the fact that glucose does not depress urea formation from injected amino acids while it does suppress urea formation from ACE. Conversely, the failure to get an additive effect when amino acids and ACE are given and the normal response to amino acids in the adrenalectomized rat argue against ACE having a significant influence on deamination of amino acids. The findings of Wells and Kendall (1940) that the adrenalectomized phlorhizinized rat fed casein alone was able to metabolize this protein and excrete the same amount of nitrogen and glucose as the intact phlorhizinized rat and that of Segalloff (1946) that the adrenalectomized rat can be maintained in good condition on a high protein carbohydrate free diet likewise support the view that the adrenalectomized rat has no serious defect in amino acid metabolism. Protein taken in the gastrointestinal

tract is, of course, introduced into the metabolic pool as amino acids. The decrease in gluconeogenesis from amino acids in kidney tissue in vitro reported by Russell and Wilhelmi (1941) may represent a special case applicable chiefly to renal metabolism. Koepf *et al.* (1941) found no such defect in liver slices from adrenalectomized rats.

However, the mechanism by which ACE increases the catabolism of whole protein is by no means clear. The fact that this response is readily inhibited by glucose and amino acids indicates that protein breakdown is not an obligatory action of ACE and suggests that it may be secondary to events occurring elsewhere in the metabolic pathways of carbohydrate and fat. There is ample experimental evidence indicating that the adrenal cortex is concerned with the utilization of carbohydrate. The studies of Long (1940, 1942) confirmed by many other investigators have indicated that in the fed animal a decreased oxidation of carbohydrate must occur to explain the accumulation of glycogen and the increase in blood sugar that occurs after treatment with adrenal cortical steroids or adrenocorticotrophic hormone. Nitrogen excretion in the fed animal or patient given adrenal cortical steroids or adrenocorticotrophic hormone varies from no change to moderate increases as the dose of hormone is increased, but in no case has been sufficient to account for the accumulation of carbohydrate that occurred (Ingle, Li, and Evans, 1946, Forsham, Thorn, Prunty, and Hills, 1948, Conn. Louis, and Wheeler, 1948). Pancreatic diabetes is alleviated by adrenalectomy (Long and Lukens, 1936). When account is taken of the decrease in food intake which follows adrenalectomy it is apparent that the improvement in the diabetes must be attributed more to increased utilization of carbohydrate in the absence of the adrenal cortex than to decreased gluconeogenesis from protein (Ingle and Prestrud, 1948).

On the basis of the data presently available, one might be tempted to speculate that the primary site of action of the adrenal cortex in metabolism is on carbohydrate metabolism, decreasing the oxidation of glucose and directing glucose disposal to glycogen storage and possibly conversion to fat. In the fasted animal this would make necessary the breakdown of protein to supply the precursors for glycogen and fat, while at the same time fat would be mobilized and oxidized to supply the calories not readily available from carbohydrate. In the fed animal the response to cortical steroids would depend on the caloric intake and the dose of hormone. Under optimal circumstances the materials supplied in the diet would obviate the need for extra endogenous protein catabolism to meet the change in metabolism. With larger doses of hormone or an indaequate dietary intake the tissue proteins might still be called on. Our observation that glucose and amino acids prevent the breakdown of protein is consistent with such a view since they would represent the substrates necessary to carry the process forward without calling on tissue protein. For

reasons already noted the results with the non-glycogenic amino acids which seem contradictory cannot be considered as conclusive. One might anticipate that other 3-carbon intermediaries such as lactate and pyruvate might be equally effective, but this experiment has not been done. The administration of fat might influence the pathway from carbohydrate to fat by the mass action law but would have little effect on glycogen synthesis. For this latter reaction another precursor, tissue proteins, would still be required. Whole protein intravenously might be anticipated to be called on in the same way as tissue protein and its utilization rendered largely unnecessary by glucose.

Although our data are readily explained by the above hypothesis there are reasons for not rejecting the possibility that the adrenal cortex may in addition play a role directly in the metabolism of protein. The adrenalectomized animal can still draw readily from protein in the diet and avoid hypoglycemia, but cannot do so readily by increasing tissue protein catabolism and gluconeogenesis, immediately after adrenalectomy, although later it can (Ingle and Oberle, 1946 Noble and Toby, 1948). The adrenalectomized-nephrectomized rat forms urea at a relatively constant rate from the time of operation to death, whereas the control nephrectomized rat catabolizes protein and makes urea at an ever increasing rate until death (Bondy and Engel, 1947). The adrenalectomized animal cannot mobilize protein in response to a stress as does the normal animal, and does not utilize injected plasma in the same way as the normal rat (Bondy, Engel, and Farrar, 1949). The adrenalectomized mouse does not draw on its lymphoid tissue nitrogen during a prolonged fast (White and Dougherty, 1948). It would appear possible that the adrenal cortex may be concerned both with the mobilization and translocation of protein and the catabolism of this protein. The magnitude of the latter reaction may depend on the relative availability of extra carbohydrate, amino acids or other precursors of carbohydrate. During fasting or when fat alone is available protein catabolism will of necessity occur, whereas if there is a plethora of glucose or amino acids protein catabolism will be inhibited. Further investigation is still necessary, however, to place clearly the protein catabolic action of the adrenal cortex in relation to the other changes in metabolism.

SUMMARY

An increase in urea formation begins 3 hours after the subcutaneous administration of adrenal cortical extract to fasted nephrectomized rats.

This increase is prevented by glucose injected intravenously three hours after the cortical extract.

Injection of a mixture of the ten essential amino acids plus glycine resulted in no greater amount of urea formation in the nephrectomized rat when given in conjunction with adrenal cortical extract than

when either the amino acids or the adrenal cortical extract alone were injected. Data are presented to suggest that the amino acids actually inhibited the breakdown of endogenous protein which usually follows the injection of adrenal cortical extract into the fasted animal.

Similar results were obtained if the amino acid mixture was modified to contain predominantly non-glycogenic amino acids or if lysine alone was injected.

Intravenous fat administration did not inhibit the increase in urea formation after adrenal cortical extract.

Human serum albumin injected at the same dose level in terms of nitrogen as the amino acids did not stimulate urea formation in the nephrectomized rat. In conjunction with adrenal cortical extract, however, a slightly greater amount of urea was formed than after cortical extract alone. This effect was abolished if glucose was given with the albumin.

These data are interpreted to indicate that (a) the action of the adrenal cortex in nitrogen metabolism is on whole protein rather than amino acids and (b) that the increase in protein catabolism after adrenal cortex extract is readily modified by changing the internal metabolic environment. It is suggested that the amount of glucose or of glycogen precursors available may be a determining factor in whether protein catabolism will be stimulated by the adrenal cortex. The interpretation of the role of the adrenal cortex in protein metabolism as entirely secondary to changes in carbohydrate and/or fat metabolism is considered but held not to be adequate in light of presently available data. The suggestion is made that the adrenal cortex may be necessary to make protein available for catabolism but that the latter reaction occurs only if no other substances capable of readily entering the metabolic pathways of carbohydrate are available.

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THE METABOLISM OF AMINO ACIDS AND PROTEIN IN THE ADRENALECTOMIZED-NEPHRECTOMIZED RAT[1]

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IN THE PRECEDING paper data were presented on the nature of the protein catabolic effect of adrenal cortical extract in nephrectomized rats (Engel, Schiller and Pentz, 1949). It was shown that the intravenous injection of glucose or amino acids 2 to 3 hours after adrenal cortical extract inhibited or masked the usual stimulation of nitrogen metabolism that occurred after administration of this hormone. It was further demonstrated that cortical hormone did not stimulate the conversion of injected amino acids to urea whereas it did stimulate urea formation after the injection of albumin. The latter effect was inhibited if glucose was given with the protein. It was concluded that in its action on protein metabolism and gluconeogenesis the adrenal cortex did not detectably influence the rate of deamination of amino acids but did intervene at the level of protein degradation itself.

The present study on the metabolism of amino acids and proteins in the adrenalectomized-nephrectomized rat was designed to throw further light on this problem. By the use of the adrenalectomized rat it is possible not only to examine the effects of the absence of the adrenal itself. but also to evaluate the possible role of non-specific stimulation of protein metabolism as a result of the experimental procedures in the animals with intact adrenals.

MATERIALS AND METHODS

Male rats from Rockland farms, weighing between 180 and 240 grams, were fed a low potassium diet (Bondy and Engel, 1947) for three days before the experiment. Bilateral nephrectomy or adrenalectomy and nephrectomy were performed, and the animals injected with 2.5 mg. of desoxycorticosterone acetate in oil subcutaneously at the time of operation and at 18 or 40 hours postoperatively. All food was withheld, but water and saline were

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offered freely. The rats were bled from the tail at the time of operation, and at 18, 21, and 24 hours, or at 40, 43, and 46 hours after operation. The blood samples were analyzed for urea by the xanthydrol precipitation method of Engel and Engel (1947). At 21 or 43 hours, the test substance was administered by intravenous injection into the internal saphenous vein, which was exposed by a skin incision. The injection took five minutes for completion. Light nembutal anesthesia was used for operation and during the period of bleedings. The rate of urea formation was calculated from the increase of the blood urea level, assuming that urea was equally distributed throughout the total body water (63% of the body weight). Rates of urea formation were expressed as milligrams nitrogen per 100 grams body weight per hour. Most of the test substances injected were the same as in the previous report and administered in the same doses (Engel, Schiller and Pentz, 1949). In addition rat plasma was prepared in the following manner: At the end of the experiments, rats were killed by decapitation and their blood collected in heparin. The plasma was separated and frozen. When an adequate amount had accumulated, it was thawed, filtered through a Seitz filter into a sterile cellophane bag, and dialyzed for 12–18 hours against cold running tap water, then against 0.5 per cent sodium chloride solution for one hour. The bag was hung in the blast of an electric fan in a cold room until the contents had concentrated to less than their original volume. The preparation was analyzed for nonprotein nitrogen and total protein, and diluted with distilled water until the concentration of protein approximated 13 mg. of protein nitrogen per ml. The material was then refiltered, and stored under aseptic conditions. One hundred units of penicillin and 1:1000 merthiolate were added. The plasma thus obtained was sterile on culture and had no pyrogens when tested in rabbits. The quantity administered was adjusted to supply 10.4 mg. of protein nitrogen per 100 grams of rat. In some experiments glucose was added to the plasma to a concentration of 6 per cent.

RESULTS

In a previous report from this laboratory (Bondy and Engel, 1947) it was shown that in order to achieve a reasonable period of survival after combined adrenalectomy and nephrectomy, it was necessary to deplete the animals of potassium for several days preoperatively. Under these circumstances the adrenalectomized-nephrectomized rat actually survived longer than the nephrectomized rat similarly pretreated with a low potassium diet and with injections of desoxycorticosterone acetate postoperatively. The nitrogen metabolism of the adrenalectomized animals was characterized by a relatively constant rate of urea accumulation from the time of operation until death whereas there was a rapidly accelerating rate in rats with intact adrenals. Since it appeared possible that the pattern of protein metabolism might be somewhat different shortly postoperatively in the rats with intact adrenals, compared to a later interval when urea formation had sharply increased, the present experiments were carried out at two intervals, namely 18 to 24 hours and 40 to 46 hours postoperatively. The average hourly rates of urea formation from

operation to the 18th and 40th hours, and from the 18th to 21st, and 40th to 43rd hours were compared in different series of nephrectomized rats with and without adrenals. During the 21st to 24th and the 43rd to 46th hours the effects of the various test materials were examined. The results of these experiments are shown in Tables 1 and 2.

TABLE 1. EFFECTS OF AMINO ACIDS, PLASMA PROTEIN, A.C.E. AND GLUCOSE ON UREA FORMATION IN NEPHRECTOMIZED AND ADRENALECTOMIZED-NEPHRECTOMIZED RATS ON LOW POTASSIUM DIET 21 HOURS POSTOPERATIVELY

Procedure	No. of rats	Rate of urea N formation, mg. N per 100 gm. body weight per hr.				
Hours postoperatively		0–18 hrs.	"p"	18–21 hrs.	"p"	21–24 hrs.
Nephrectomized VuJ-n* (9.4 mg. N per 100 gm.)	16	2.7±0.11	<0.01	1.8±0.18	<0.01	3.0±0.32
"p"		<0.02		N.S.		N.S.
Adrenalectomized-nephrectomized VuJ-n* (9.4 mg. N per 100 gm.)	17	2.3±0.11	<0.01	1.3±0.18	<0.01	2.9±0.35
Nephrectomized plasma* (10.4 mg. N per 100 gm.)	6	2.4±0.13	<0.01	1.6±0.24	<0.02	2.6±0.24
"p"		N.S.		<0.01		<0.01
Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gm.)	7	2.5±0.16	<0.01	0.7±0.12	N.S.	0.8±0.30
"p"		N.S.		N.S.		<0.02
Adrenalectomized-nephrectomized ACE† (0.8 ml. per 100 gm.) plasma* (10.4 mg. N per 100 gm.)	8	2.3±0.15	<0.01	1.0±0.18	<0.01	2.1±0.35
Adrenalectomized-nephrectomized ACE† (0.8 ml. per 100 gm.)	3‡	2.6		2.2		2.5
Nephrectomized normal potassium diet. Plasma* (10.4 mg. N per 100 gm.)	7			2.3±0.31	N.S.	2.6±0.32
Nephrectomized glucose* (50 mg. per 100 gms.)	12	2.9±0.14	<0.01	1.8±0.13	N.S.	1.7±0.32
"p"		N.S.		N.S.		N.S.
Adrenalectomized-nephrectomized glucose* (50 mg. per 100 gms.)	10	2.7±0.10	N.S.	1.7±0.45	N.S.	1.5±0.36

* Administered intravenously at the 21st hour.
† Administered subcutaneously at the 18th and 19th hours.
‡ Number of animals too small for statistical analysis.

The rate of urea production immediately after operation was higher than the rate from 18–21 hours. The change was significant in all groups except in the adrenalectomized rats injected with glucose at 21 hours. In this series, the decrease of the mean was as large as in some of the other series, but the large standard error of the 18–21 hour period brought the value of "p" higher than 0.05. The higher postoperative rate of urea production may be related to the trauma of operation. However, it is of interest that this higher rate was not observed in rats having a normal potassium intake in the diet (Engel, Pentz, and Engel, 1948).

After the injection of the amino acid mixture, VuJ-n, there was a significant increase in the rate of urea production both in animals with and without adrenals, in the 18 hour series. The increment was slightly larger in the adrenalectomized group, possibly because of the

somewhat lower initial (18–21 hour) rate of urea production in this series. No significant difference was found between the adrenalectomized animals and those with intact adrenals in their ability to increase their rate of urea production after the administration of amino acids. The final rates of urea formation in rats with and without adrenals were identical. It may be noted that the increment produced by VuJ-n in this experiment is the same as that previously reported in a different series of animals but on a chow diet and with intact adrenals, similarly injected in this laboratory (Engel, Pentz and Engel, 1948). These results confirm Evans' finding (1941) that the adrenalectomized-nephrectomized rat shows no impairment of ability to deaminate injected alanine.

The above results are in contrast to those obtained when the nitrogen was injected in the form of whole protein (rat plasma). After the administration of rat plasma there was no change in the rate of formation of urea in the adrenalectomized animals. Animals with intact adrenals, however, increased their rate of urea production significantly. The rate of urea formation was increased from 1.6 ± 0.24 in the control period to 2.6 ± 0.24 mg. per 100 gm. body weight per hour during the three hours following the injection of plasma. There was a significant difference in the rate of production of urea after the injection of plasma in adrenalectomized as compared to non-adrenalectomized animals. The increase in urea formation after plasma injection into nephrectomized rats with intact adrenals differs from the results described in the previous report (Engel, Schiller and Pentz, 1949). No change in urea formation was noted after human serum albumin treatment in rats similarly nephrectomized but previously maintained on a diet adequate in potassium unless adrenal cortical extract was given. The studies in this laboratory were suspended before it was possible to test the effect of albumin in the above strain of rat on a low potassium diet. However, one of us (F.L.E.) has studied the effect of intravenous plasma on the rate of urea formation in nephrectomized rats on a diet of normal potassium content in the Duke laboratory. The rate of urea formation was 2.3 ± 0.31 mg. per 100 gms. body weight prior to plasma injection and 2.6 ± 0.32 mg. per 100 gms. following treatment, an insignificant change (Table 1). This corresponds to the results with albumin in the same strain of rats under the same experimental conditions. It is conceivable, therefore, that the potassium deficiency is responsible in some way for the increase in urea formation after plasma.

These results imply that the adrenal cortex is necessary for the increase in the rate of urea production in fasting potassium depleted rats after the injection of rat plasma. In order to confirm this hypothesis, adrenalectomized-nephrectomized rats were injected with adrenal cortical extract 2 and 3 hours before the injection of plasma. In Table 1 it may be seen that, under these circumstances, there was

a significant increase in the rate of production of urea after the injection of plasma, the rate increasing from 1.0 ± 0.18 to 2.1 ± 0.35 mg. per 100 gm. body weight per hour. No significant differences were found between the rates of urea formation in the rat with intact adrenals and the adrenalectomized rat given adrenal cortical extract. The difference in the rate of urea formation after the administration of adrenal cortical extract and plasma to the adrenalectomized rat was significant as compared to the response to plasma alone, without the hormone.

It was desirable to separate the effect of adrenal cortical extract on the rate of urea synthesis in the adrenalectomized rat from the effect of the injection of plasma. A series of adrenalectomized rats was therefore given adrenal cortical extract without plasma; however limitations of time did not permit finishing this series. The incomplete results are shown in Table 1. It appears that the effect of the injection of adrenal cortical extract into an adrenalectomized rat is intermediate between the result when plasma alone is administered, and when both plasma and adrenal cortical extract were given.

A series of adrenalectomized-nephrectomized and nephrectomized rats were injected at 21 hours with glucose . This experiment did not produce any significant alteration in the rate of urea production in either group. It may therefore be accepted as a control observation, indicating that the handling of the rats and the injection of the volumes of fluid involved in the present experiments do not themselves influence the rate of urea synthesis. It may be noted that the lack of

TABLE 2. EFFECTS OF AMINO ACIDS, PLASMA AND GLUCOSE ON UREA FORMATION IN NEPHRECTOMIZED AND ADRENALECTOMIZED-NEPHRECTOMIZED RATS ON A LOW POTASSIUM DIET, 40 HOURS POSTOPERATIVELY

Procedure	No. of rats	Rate of urea N formation mg. N per 100 gms. body weight per hr.				
Hours postoperatively		0–40 hrs.	"p"	40–43 hrs.	"p"	43–46 hrs.
Nephrectomized VuJ-n* (9.6 mg. N per 100 gm.)	17	2.9 ± 0.13	N.S.	3.2 ± 0.14	N.S.	3.8 ± 0.49
"p"		<0.01		<0.01		N.S.
Adrenalectomized nephrectomized VuJ-n* (9.6 mg. N per 100 gm.)	15	2.3 ± 0.14	N.S.	2.2 ± 0.28	<0.05	3.4 ± 0.52
Nephrectomized plasma* (10.4 mg. N per 100 gm.)	8	2.3 ± 0.07	N.S.	2.2 ± 0.24	<0.01	3.6 ± 0.31†
"p"		N.S.		N.S.		<0.01
Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gm.)	6	2.2 ± 0.14	N.S.	1.9 ± 0.61	N.S.	1.5 ± 0.30
Nephrectomized plasma* (10.4 mg. N per 100 gms. in 5% glucose	7	2.3 ± 0.12	N.S.	2.9 ± 0.45	<0.01	1.4 ± 0.12†
"p"		<0.01		<0.02		<0.01
Adrenalectomized-nephrectomized plasma* (10.4 mg. N per 100 gms. in 5% glucose	6	1.6 ± 0.07	N.S.	1.2 ± 0.41	N.S.	0.7 ± 0.19

* Administered intravenously at the 43rd hour.
† "p" value <0.01 for the nephrectomized animals given plasma compared to the nephrectomized animals given plasma in 5% glucose.

response to this dose of glucose is consistent with previously reported results (Engel, Schiller and Pentz, 1949).

Table 2 records the results of experiments carried out 40–46 hours postoperatively. In this series the animals were usually quite ill during the experiment, so that the results obtained were not so uniform as in the 18 hour group.

After the injection of amino acids (VuJ-n) there was an increase in the rate of urea formation in the adrenalectomized animals. The increment, 1.2 mg. per 100 gm. per hour is comparable with 1.6 mg. per 100 gm. per hour observed 18 hours after operation. In animals with intact adrenals there was also an average increase in the rate of urea formation, but the rise was small (0.6 mg. per 100 gm. per hour) and not statistically significant. The lack of significance can be related in part to the large standard errors obtained in this series, when the animals were within a few hours of death. The larger response of the adrenalectomized animals may be due in part to the lower basal urea formation rate, and in part to the fact that adrenalectomized potassium-depleted animals develop uremia more slowly than do those with intact adrenals (Bondy and Engel, 1947) so that their condition was less desperate than that of the rats with intact adrenals at this time.

The injection of plasma was followed by no increase in the rate of urea formation in the animals without adrenals, but in those whose adrenals were intact, there was a rise which was comparable to that obtained at 18 hours, and considerably larger than that shown at 40 hours after the injection of VuJ-n. There was a significant difference in the rate of urea formation after the injection of plasma in the adrenalectomized rats as compared with those with intact adrenals. The response of the animals to VuJ-n and to plasma was similar at 18 and 40 hours postoperatively, suggesting that the effects of adrenalectomy are well established 18 hours after operation.

The extra urea formation when albumin was given to ACE treated rats was prevented if glucose was administered with the albumin (Engel, Schiller, and Pentz, 1949). It seemed of interest to determine whether the increase in urea after plasma which occurs in potassium deficient nephrectomized rats even in the absence of extra cortical hormone could be blocked by glucose. As seen in Table 2, the simultaneous administration of glucose and plasma produced a fall in the rate of urea formation in both adrenalectomized and non-adrenalectomized animals. The reduction in the rate of urea formation was significant in the animals with intact adrenals. It should be noted that glucose not only totally blocked the effect of the injection of plasma on the urea production rate of animals with intact adrenals but also reduced the higher rate of urea formation occurring at 40 hours as compared to 21 hours after nephrectomy. Thus the increases in urea formation after plasma and after the stress of increasing ure-

mia in rats with intact adrenals are subject to the same inhibitory influence from glucose as are those after excess ACE, with or without albumin.

DISCUSSION

The response of the adrenalectomized rats to the administration of amino acids supports the point of view that the adrenal cortical hormones do not promote deamination of amino acids. It is apparent that, under the circumstances of the experiment, adrenalectomized rats were capable of deaminating amino acids as well as rats with intact adrenals. Evans (1941) had previously reported observations which agree with this finding. He showed that the injection of dl-alanine was followed by an increase of urea production in adrenalectomized-nephrectomized rats which was equal to that found in normal controls.

An explanation is not apparent for the increase in urea formation after plasma in the potassium-depleted nephrectomized rats compared to the lack of response to plasma or albumin in the presence of an adequate potassium intake. Conceivably it might be due to a decreased ability to store protein intracellularly in the face of a potassium deficiency. However, the possibility cannot be ruled out that, despite all precautions, this plasma used in these experiments was toxic and stimulated endogenous protein catabolism. In any case the failure of the adrenalectomized-nephrectomized rat to show an increase in urea formation after plasma unless cortical extract is given is significant. It indicates that the adrenal cortex is necessary for the increase in nitrogen metabolism that occurs after plasma although there is no proof here that it is responsible for it. This is similar to and may be even identical with, the relation of the adrenal cortex to the increased nitrogen excretion after stress. Ingle, Ward, and Kuizenga (1947) have shown that the adrenal cortex is necessary, but not responsible for this response. Our previous observation (Bondy and Engel, 1947) that the rate of urea formation in adrenalectomized rats remains constant from nephrectomy to death unless cortical extract is given, whereas it accelerates from day to day in rats with intact adrenals is another example of the role of the adrenal in the mobilization and catabolism of protein after stress. The increase in urea formation after plasma as well as the higher rate of urea formation 40 hours after nephrectomy were both modified by glucose just as was the stimulation of protein catabolism by adrenal cortical extract (Engel, Schiller, and Pentz, 1949). Since glucose had no effect on deamination of amino acids these results suggest that glucose is acting at the same level in all these cases, i.e. inhibition of protein breakdown itself.

When taken in conjunction with the normal rate of urea formation after amino acid injection in the adrenalectomized rats and the ob-

servations described in the previous report, the results with plasma are consistent with the view that the adrenal cortex intervenes in protein metabolism at the level of protein breakdown rather than elsewhere. Where there is the element of stress the action of the adrenal cortex in protein catabolism apparently is a secondary one and is linked to some other as yet unidentified factor.

SUMMARY

The rates of urea formation in nephrectomized and adrenalectomized-nephrectomized rats previously on low potassium diets have been investigated as measures of nitrogen metabolism. Studies were conducted at 18 and 40 hours postoperatively.

The adrenalectomized-nephrectomized animals formed urea at the same rate as did the controls during the first 21 hours postoperatively but generally at a lower rate after 40 hours.

The adrenalectomized-nephrectomized rats formed urea at the same rate after an injection of amino acids as did the control nephrectomized rats.

The adrenalectomized-nephrectomized rats showed no increase in urea formation after injection of rat plasma, but did if adrenal cortical extract was given 2 and 3 hours before the plasma. The control nephrectomized rats responded to intravenous plasma injection by a significant increase in urea formation. The latter was prevented if glucose was given with the plasma.

Glucose intravenously had no effect on the rates of urea formation in either group of rats at 18 hours postoperatively. At 40 hours postoperatively it caused a significant decrease in urea formation in the nephrectomized rats with intact adrenals.

The data are interpreted to support the view that the action of the adrenal cortex in protein metabolism is at the level of protein break down itself rather than at the level of deamination of amino acids, regardless of whether this be a direct or an indirect action of the hormone.

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NOTES AND COMMENTS

THE THYROXINE-LIKE ACTION OF TETRABROM-THYRONINE

Abderhalden and Wertheimer (1928) and Gaddum (1929–30) have shown that 3:5:3′:5′-tetrabrom-thyronine, the bromine analogue of thyroxine, raises the metabolic rate of the rat when given in doses of twenty to fifty mgms. per kilo. It has now been shown that this substance in doses of 50 μg. per 100 gm. per day prevented the development of thyroid and pituitary changes in rats receiving methylthiouracil. The thyroids showed subnormal activity with completely flattened epithelium and colloid storage. The pituitary basophils were normal in appearance and number (8%). Tetrabrom-thyronine has, therefore, about one twentieth the biological activity of DL-thyroxine. As in the case of thyroxine, the amount necessary to prevent pituitary and thyroid changes is about one one hundredth of that necessary to produce a distinct increase in the metabolic rate.

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NEW BOOKS

PREGNANCY DIAGNOSIS TESTS: A REVIEW. By Alfred T. Cowie. Published by the Commonwealth Agricultural Bureaux Joint Publication No. 13. Shinfield, Reading, Great Britain, 1948. 283 pp. 15s.

This is a most useful book. The author, of the National Institute for Research in Dairying, has searched the literature on pregnancy tests applicable to all species and has reviewed it in a most readable and interesting volume. Species differences plague the student of reproduction more vexatiously than in any other field of physiology. A sure-footed survey of the comparative aspects of even a part of this field is therefore welcome.

The various pregnancy tests, and they are legion, have been classed into seven main groups. They are the tests based on:

1. clinical methods
2. hormonal investigations of body fluids
3. enzymic investigations of body fluids
4. other biochemical investigations of body fluids
5. physiological phenomena
6. immunological phenomena
7. physical investigations of body fluids.

These chapters are rounded out by a brief but informative summary and conclusions. The bibliography contains nearly two thousand titles, and the book includes a well designed index.

Although, as is inevitable, woman receives her lion's share of attention, it is startling to find no mention of her in the chapter on clinical methods. It is true that such an omission is justified by the numerous textbooks of obstetrics that are available: nevertheless even a short account would have completed the section. Except for this one omission, the physiologist, the veterinarian and the physician alike will read the volume with interest and profit.

THE EPITHELIA OF WOMAN'S REPRODUCTIVE ORGANS. By George N. Papanicolaou, Herbert F. Traut and Andrew A. Marchetti. Published by the Commonwealth Fund, New York, 1948. vi+53 pp. Price $10.00.

The reproductive cycle of female mammals is accompanied by extensive changes in the morphology and physiology of the cells and tissues comprising the reproductive organs. The fact that cyclical variations exist has been recognized for long periods, but the accurate timing of the reproductive cycle, essential for any accurate study of the changes, became possible only during the last three decades. In 1917, Stockard and Papanicolaou recognized that the cytological study of vaginal smears provided an accurate end-point for studies of the reproductive cycle. This discovery ushered in a remarkable series of investigations clarifying the temporal sequences of the cycle in a number of mammalian species including man and leading directly to the further discoveries of the physiological effects and chemical natures of the ovarian hormones.

Despite the rapid increase in knowledge, the study of human reproduction has lagged behind that of the lower species. To a considerable extent, our concept of the human reproductive cycle represents inferences made by homology with the corresponding events as observed in animal experiments. Detailed information on the human species has been slow forthcoming. Studies such as those of Hertig and Rock on early human embryos, Papanicolaou on the human vaginal smear and now on the reproductive organs are therefore all the more welcome.

In the introduction, the authors state that:

"This monograph is not intended to take the place of a handbook. The interpretations given here largely reflect our own views, and some of them may be found to conflict with prevailing concepts. In our endeavor we have been guided by the desire to stimulate more thought in certain directions and to point out the growing importance of the cytologic approach."

Their account does indeed do violence to occasional preconceptions, especially those based on experiments on lower forms. Some species of monkeys have tough, fibrous adhesions of the ovary, induced by the prevailing filarial infections, which apparently do not hinder ovulation in the slightest. The present authors, on the contrary, state that a thick fibrous capsule hinders and prevents ovulation. The contents of the uterine lumen and the uterine glands may contain copious amounts of glycogen, according to the present study, but no attention is given to the possibility that this material may represent mucus, which spuriously gives many of the cytological reactions for glycogen. By analogy with the lower animals, mucus should be suspected in this location and recent investigations have proved its presence there in the human. The question remains, therefore, whether both glycogen and mucus occur in the secretions of the glandular lumina, or whether mucus alone can account for all of the observations.

The criticisms above suggest that in their zeal to present the most accurate and painstaking record of the cytology of the human reproductive tract, perhaps too little attention has been devoted to homologous situations. But this is a minor matter—the positive value of the detailed cytological information presented greatly outweighs the minor negative criticisms.

No review of this book could be complete without mention of the twenty-one plates of colored illustrations which document it. Some are semidiagrammatic, representing histological interpretations, some are drawings and some are photomicrographs. All are carefully and beautifully printed. It is unfortunate that a few of the figures appear to be retouched without appropriate mention of the fact, and that plate 16 is mislabeled a photomicrograph when it is actually a drawing. Nevertheless, they represent the highest expression of the art of the histologist and the color-printer.